ENGINEERED TERMINAL NUCLEOTIDYL TRANSFERASE VARIANTS

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0001] This application claims the benefit of U.S. Provisional Application No. 63/634,824, filed April 16, 2024, and U.S. Provisional Application 63/706,470, filed October 11, 2024, all of which are incorporated by reference herein.

TECHNICAL FIELD

[0002] The present invention provides engineered terminal nucleotidyl transferase (TnT) polypeptides useful in template-independent polynucleotide synthesis, as well as compositions and methods of utilizing these engineered polypeptides.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0003] The official copy of the Sequence Listing is submitted concurrently with the specification as an XML file, with a file name of "CX10-267WO3_ST26.xml," a creation date of April 16, 2025, and a size of 7,615,530 bytes. The Sequence Listing filed is part of the specification and is incorporated in its entirety by reference herein.

BACKGROUND

[0004] Synthetic biology is becoming established in a diverse range of high value, high growth markets. From food and agriculture to therapeutics, diagnostics, and vaccines; tools such as gene editing, DNA sequencing and gene synthesis are being used to build value-added products with advanced functionality (e.g., cell bioreactors, etc.) and desired end products (e.g., drugs, chemicals, etc.). The barrier to widespread implementation of these technologies is the ability to efficiently synthesize RNA, DNA, and other polynucleotides.

[0005] In particular, silencing RNA (siRNA) therapeutics are a promising class of drugs that have the potential to treat numerous difficult to treat conditions in a highly targeted manner by binding to known mRNA targets (Hu et al. (2020). Sig Transduct Target Ther 5, 101; Zhang et al. (2021). Bioch. Pharmac., 189, 114432.) As these therapies become more common and are targeted at larger patient populations, the ability to produce large amounts of the oligonucleotide active pharmaceutical ingredient (API) becomes critical.

[0006] To date, short RNA oligonucleotides have been synthesized almost exclusively by iterative addition of nucleotides in the form of activated phosphoramidites, plus additional processing steps, to a growing immobilized nucleotide chain (Brown, T. Nucleic Acids Book. See at: www.atdbio.com/nucleic-acids-book (accessed 2022-10-10).)

[0007] Phosphoramidite chemistry has been developed extensively over the years to synthesize small amounts of DNA and for more complex therapeutic RNA syntheses but suffers from several cost and sustainability issues that are potentially limiting as API demand grows to triple-quadruple digit kilograms per year (Andrews et al. (2021). J. Org. Chem. 86, 49–61). Additionally, RNA synthesis using phosphoramidite

synthesis chemistry is limited to producing short oligonucleotides of approximately 200 basepairs (Beaucage & Caruthers. (1981). Tetrahedron Lett. 22 (20): 1859.)

[0008] The phosphoramidite iterative methodology is multi-step and based on phosphorous (III) coupling chemistry that requires (i) coupling (ii) capping (iii) oxidation to P(V) forming phosphodiester or phosphorothioate diester (iv) deblocking of 5'-O group. After chain synthesis is complete, the final oligo is cleaved from the support where deblocking of phosphate cyanoethyl group and nucleobases can also occur (Brown, T. Nucleic Acids Book. See at: www.atdbio.com/nucleic-acids-book (accessed 2022-10-10).) Washes with organic solvents at each step are also required. The phosphate cyanoethyl blocking group and nucleobase protecting groups can be removed in parallel to oligonucleotide cleavage from the solid support to generate the oligonucleotide product, or the cyanoethyl group can be removed under milder condition before chain cleavage, if required.

[0009] Many aspects of the environmental impact of the current phosphoramidite methodology and potential advances have been reviewed (Andrews et al. (2021). J. Org. Chem. 86, 49–61). Even at an aspirational high oligonucleotide loading of 20% mass final oligonucleotide to mass solid support, at least five-fold mass of support is required over the final product mass.

[0010] In addition to the high cost of mass support required to immobilize the oligonucleotide, the use of organic solvents and 5'-O-blocking groups entail additional waste and process inefficiencies. Organic solvents such as acetonitrile are required for solubilization of the phosphoramidite coupling partners, or dichloromethane or toluene for deprotection steps. These solvents need to be anhydrous to reduce undesired hydrolysis of the phosphoramidite partners and can come from non-sustainable sources, adding cost, sustainability questions, and potential supply issues to the process.

[0011] The phosphoramidite coupling partners themselves carry a required blocking group at the 5'O-position, the nucleobase nitrogen atom (in A, C and G), and the nascent phosphate. The most common 5'O-blocking group, dimethoxytrityl, has a molecular mass of ~ 303 Da that approaches that of the heaviest native ribonucleotide fragment Gp with a mass of ~ 345 Da. This protecting group requires energy, resources, and effort to produce and append, and then requires disposal when separated from the desired materials.

[0012] In conclusion, a paradigm shift in oligonucleotide synthesis is necessary to enable siRNA therapeutics by lowering environmental impact, improving economic efficiency, and increasing scalability. New methods of oligonucleotide synthesis are, therefore, of great interest to the pharmaceutical industry.

Template-Independent Enzymatic Synthesis

[0013] Enzymatic synthesis may facilitate production of high volumes of complex or long polynucleotides (>200 base pairs) while minimizing toxic waste. A variety of prokaryotic and eukaryotic DNA and RNA polymerases are known to naturally synthesize polynucleotides of thousands of base pairs or more. Most of these polymerases function during DNA replication associated with cell division or transcription of RNA from DNA associated with gene or protein expression. Both of these processes involve template-dependent polynucleotide synthesis, wherein the polymerase uses an existing template polynucleotide strand to synthesize a complementary polynucleotide strand.

[0014] The potential of template-independent enzymatic polynucleotide synthesis to produce defined sequences has long been recognized. One early report suggested using NTPs with blocked 3' groups to allow stepwise addition of specific nucleotide residues (Bollum, J Biol Chem, 1962, 237, 1945–1949).

[0015] However, few polymerases are known to catalyze template-independent polynucleotide synthesis. These include polymerase lambda, polymerase mu, and terminal deoxynucleotidyl transferase (TdT), all members of the X family of DNA polymerases, many of which participate in DNA repair processes (Domínguez et al., EMBO, 2000, 19(7), 1731–1742.) Of these, TdT is known to generate diversity in antigen receptors by indiscriminately adding nucleosides to the 3' end of a single-stranded polynucleotide in a template-independent process (Bentolila et al., EMBO, 1995, 14(17), 4221–4229.)

[0016] Others have published a method of polynucleotide synthesis using a nucleoside 5'-triphosphate with a 3'-OH position protected with a removable blocking moiety and, specifically, a template-independent polynucleotide polymerase, including a terminal deoxynucleotidyl transferase (U.S. Pat. 5,763,594). The blocking group, also known to those skilled in the art as an inhibitor or reversible terminating group, may include a variety of groups that prevent the TdT from adding additional NTPs to the nascent polynucleotide chain. This may include charged molecules, large molecules and moieties, or other blocking groups known to those skilled in the art. Appropriate removable blocking groups may include carbonitriles, phosphates, carbonates, carbamates, esters, ethers, borates, nitrates, sugars, phosphoramidates, phenylsulfenates, and sulfates. Other 3' blocking groups are also known in the art, including 3'-O-amines and methylamines (U.S. Pat. 7,544,794) and 3'-O-azides (U.S. Pat. 10,407,721).

[0017] Although initially promising, use of 3'-blocked NTPs in template-independent synthesis catalyzed by TdT has proven difficult in practice, as TdT struggles to accept 3'-O-blocked NTPs as substrates. Further, wild-type TdTs have low tolerance for oligo acceptor substrates containing one or more modified nucleotides (e.g., 2' modifications).

[0018] Additionally, synthesis of RNA strands presents unique challenges due to the additional, reactive 2'-OH on the ribose. While protection of the 2' position facilitates RNA synthesis, this approach reduces efficiency because of steric hindrance by the 2' protecting groups and requires maintenance and removal of the protecting group (CB Reese, Org Biomol Chem., 2005, 3, 3851–3868.)

[0019] Recently several reports have described template-independent synthesis methods that use modified NTPs with blocking groups attached to the purine or pyrimidine base, leaving the 3'-OH unmodified and available for additional rounds of synthesis. These base blocking groups may include a cleavable linker that allows removal of the blocking group after each NTP addition step. The cleavable linker may also be attached to a detectable label (U.S. Pat. 7,057,026, among others). A variety of cleavable linkers are known to those skilled in the art. These include linkers attached via reducible disulfide bonds, photocleavable, electrophilic or nucleophilic, pH sensitive, temperature sensitive, and linkers cleaved by enzymes. One drawback to using cleavable linkers is that, typically, some atoms of the linker moiety remain attached to the NTP following cleavage, leaving a "scar" that may interfere with synthesis of a complementary strand after initial template-independent synthesis of the primary polynucleotide strand.

[0020] Recently, modified NTPs with bases attached to blocking groups with cleavable linkers that are "scarless" and leave the nascent DNA ready for the next round of synthesis have been developed. In one example, the blocking group and cleavable linker are attached to the base via a disulfide bond. Upon addition of a reducing agent, the blocking group is removed, and the remaining atoms of the linker self-cyclize to leave the nascent DNA free of any linker atoms (U.S. Pat. 8,808,989, U.S. Pat. 9,695,470, U.S. Pat. 10,041,110). Methods of using NTPs attached to cleavable blocking groups to synthesize polynucleotides are known, including using a microfluidic device or ink jet printing technology (U.S. Pat. 9,279,149). An exonuclease may also be used in a method to synthesize polynucleotides to shorten or completely degrade polynucleotide strands that have not successfully added an NTP after the polynucleotide extension step and prior to removing the blocking group (U.S. Pat. 9,771,613).

[0021] However, NTP bases with bulky blocking groups attached via cleavable linkers are not optimal for efficient synthesis of complex or long oligonucleotides. The large labels may negatively impact enzyme kinetics, and linker scars may lead to an unacceptable rate of misincorporation when synthesizing the oligonucleotide strand. Additionally, larger linkers and necessary deblocking steps may increase the cost, time, and inefficiency of the process as a whole, rendering these methods economically infeasible.

[0022] Recently, several groups have explored modifying the structure or amino acid sequence of TdT or other polymerases to allow template-independent synthesis using 3'-O-blocked groups. Efcavitch et al. describes incorporation of 3' modified dNTPs by TdT in template-independent synthesis using a murine or bacterial TdT with substituted amino acid residues (U.S. Pat. 10,059,929). Other reports describe engineered bovine and gar (*Lepisosteus oculatus*) TdTs that displayed improved activity over wild-type TdT (U.S. Pat. 10,745,727, PCT/GB2020/050247). Similarly, a variety of mutations have been described to improve the activity of Pol X family enzymes (WO 2017216472 A2). Finally, an N-terminal truncation of the BRCT domain (or alternatively mutation of the BRCT domain) of TdT has also been described as enhancing activity in the addition of reversibly blocked NTPs to the 3'-OH of a nucleic acid (US20210164008A1).

[0023] Feasible methods of template-independent enzymatic synthesis of complex or long polynucleotides have recognized commercial value. Recently, research efforts devoted to resolving challenges in this field have yielded improved engineered enzymes with activity in the template independent synthesis of polynucleotides, including DNA (PCT/US2022/078071) and RNA (PCT/US2023/076667). However, engineered terminal nucleotidyl transferase (TnT) enzymes with further improvements are necessary to enable template-independent enzymatic synthesis of a variety of complex or long polynucleotides. In particular, TnT enzymes with improved activity under industrial process conditions that are able to accept and incorporate NTPs with an extensive range of modifications to highly modified polynucleotide oligo acceptor substrates are necessary to enable economically feasible synthesis processes.

SUMMARY

[0024] The present invention provides engineered terminal nucleotidyl transferase (TnT) polypeptides useful in template-independent polynucleotide synthesis, as well as compositions and methods of utilizing these engineered polypeptides. The TnTs of the present invention are variants of a previously engineered TnT

enzyme (PCT/US2023/076667), which is an engineered variant of a predicted splice variant of the wild-type gene from *Monodelphis domestica*.

[0025] As used herein, the term terminal nucleotidyl transferase (TnT) is used to distinguish an engineered enzyme with activity on a variety of nucleoside triphosphates, including ribonucleoside triphosphates with 3' modifications or with 2' modifications or with 2' and 3' modifications, from a wild-type TdT enzyme having wild-type TdT activity.

[0026] These engineered TnTs are capable of adding nucleoside triphosphates with a 3'-O-removable blocking group and other natural or modified NTPs to the 3'-OH end of a growing oligonucleotide or polynucleotide chain in a template-independent manner. After removal of the blocking group, additional rounds of NTP addition can be used to synthesize a polynucleotide with a defined sequence of bases without using a complementary template strand as a guide for NTP incorporation (template-independent synthesis).

[0027] In some embodiments, the present invention provides an engineered TnT polypeptide comprising an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of an even-numbered SEQ ID NO. of SEQ ID NOs: 2, 4-1056, 1130-1880, and 1928-3008, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 2, 4-1056, 1130-1880, and comprising an amino acid residue difference (e.g., at least one or one substitution set) at one or more amino acid positions, wherein the positions are numbered with reference to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and an improved property described herein.

[0028] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, sequence identity to a reference sequence of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002 and comprising an amino acid residue difference (e.g., one substitution or one substitution set) at one or more amino acid positions, wherein the positions are numbered with reference to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and an improved property described herein.

[0029] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291,

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[0030] In some embodiments, the engineered TnT polypeptide has improved thermostability, increased activity at elevated temperatures, increased soluble expression or isolated protein yield, decreased by-product formation, increased specific activity on NTP-3'-O-RBG and other natural or modified NTP substrates, and/or increased activity on various oligo acceptor substrates as compared to a wild-type TnT or other TnTs or template-independent polymerases known to those of skill in the art. These engineered TnT polypeptides with one or more amino acid substitutions or substitution sets are described, below, in the detailed description of the invention.

[0031] In some additional embodiments, the engineered terminal nucleotidyl transferase polypeptide comprises an amino acid sequence with at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of an even-numbered sequence set forth in SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008, or to a reference sequence corresponding to an even-numbered sequence set forth in SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0032] In some embodiments, the engineered terminal nucleotidyl transferase polypeptide comprises an amino acid sequence comprising residues 12 to carboxy terminal of an even-numbered sequence of an even-numbered sequence set forth in SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008, or an amino acid sequence comprising an even-numbered sequence set forth in SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0033] In some additional embodiments, the engineered terminal nucleotidyl transferase polypeptide comprises an amino acid sequence with at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562,

2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0034] In some embodiments, the engineered terminal nucleotidyl transferase polypeptide comprises an amino acid sequence comprising amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or an amino acid sequence comprising SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0035] In an additional embodiment, the engineered polypeptide of the present invention further comprises an N-terminal truncation of 1-156 amino acids of the polypeptide sequence relative to any even-numbered sequence set forth in SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0036] In an additional embodiment, the engineered polypeptide of the present invention is immobilized.

[0037] The present invention also provides an engineered polynucleotide encoding at least one engineered polypeptide described in the above paragraphs and as disclosed herein.

[0038] In some embodiments, the engineered polynucleotide comprises a polynucleotide sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 34 to 3'-terminus of SEQ ID NO: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2753, 2943, 2952, 2977, 3041, 3065, 3125, 3201, or 3227, or to a reference polynucleotide sequence corresponding to SEQ ID NO: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2753, 2943, 2952, 2977, 3041, 3065, 3125, 3201, or 3227, wherein the polynucleotide sequence encodes a terminal nucleotidyl transferase.

[0039] In some embodiments, the engineered polynucleotide comprises a polynucleotide sequence polynucleotide sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 34 to 3'-terminus of an odd numbered SEQ ID NO. of SEQ ID NOs: 3-1055, 1129-1879, and 1927-3007, or to a reference polynucleotide sequence corresponding to an odd numbered SEQ ID NO. of SEQ ID NOs: 3-1055, 1129-1879, and 1927-3007, wherein the polynucleotide sequence encodes a terminal nucleotidyl transferase.

[0040] In some embodiments, the engineered polynucleotide comprises polynucleotide sequence comprising nucleotide residues 34 to 3'-terminus of an odd-numbered sequence set forth in SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007, or a polynucleotide sequence comprising an odd-numbered sequence set forth in SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007.

[0041] In another aspect, the present invention further provides vectors comprising at least one engineered polynucleotide described above. In some embodiments, the vectors further comprise at least one control sequence.

[0042] In a further aspect, the present invention also provides host cells comprising the vectors provided herein. In some embodiments, the host cell produces at least one engineered polypeptide provided herein.

[0043] In a further aspect, the present invention provides methods of producing an engineered TnT polypeptide, comprising the steps of culturing the host cell provided herein under conditions such that the engineered polynucleotide is expressed, and the engineered polypeptide is produced. In some embodiments, the methods further comprise the step of recovering the engineered polypeptide.

[0044] In another aspect, the present disclosure provides a method of template independent oligonucleotide synthesis, the method comprising contacting an oligonucleotide acceptor substrate with an engineered terminal nucleotidyl transferase described herein in presence of at least a nucleotide triphosphate under reaction conditions sufficient for addition of a nucleotide to the 3'-end of the oligonucleotide acceptor substrate.

[0045] In some embodiments, the nucleotide triphosphate comprises a modified nucleotide trisphosphate, where the modification is at the sugar moiety, nucleobase, and/or phosphate group. In some embodiments of the method the modified nucleotide triphosphate comprises a 3'-reversible blocking group (RGB).

DESCRIPTION OF THE INVENTION

[0046] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein, and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well-known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0047] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the invention as a whole.

[0048] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the present invention. The section headings used herein are for organizational purposes only and not to be construed as limiting the subject

matter described. Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0049] As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes more than one polypeptide. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0050] It is to be understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of." It is to be further understood that where descriptions of various embodiments use the term "optional" or "optionally" the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. It is to be understood that both the foregoing general description, and the following detailed description are exemplary and explanatory only and are not restrictive of this disclosure. The section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described.

Abbreviations

[0051] The abbreviations used for the genetically encoded amino acids are conventional and are as follows:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0052] When the three-letter abbreviations are used, unless specifically preceded by an "L" or a "D" or clear from the context in which the abbreviation is used, the amino acid may be in either the L- or D-configuration about α-carbon (C_α). For example, whereas "Ala" designates alanine without specifying the configuration about the α-carbon, "D-Ala" and "L-Ala" designate D-alanine and L-alanine, respectively.

[0053] When the one-letter abbreviations are used, upper case letters designate amino acids in the L-configuration about the α-carbon and lower-case letters designate amino acids in the D-configuration about the α-carbon. For example, "A" designates L-alanine and "a" designates D-alanine. When polypeptide sequences are presented as a string of one-letter or three-letter abbreviations (or mixtures thereof), the sequences are presented in the amino (N) to carboxy (C) direction in accordance with common convention.

[0054] The abbreviations used for the genetically encoding nucleosides are conventional and are as follows: adenosine (A); guanosine (G); cytidine (C); thymidine (T); and uridine (U). These abbreviations are also used interchangeably for nucleosides and nucleotides (nucleosides with one or more phosphate groups). Unless specifically delineated, the abbreviated nucleosides or nucleotides may be either ribonucleosides (or ribonucleotides) or 2'-deoxyribonucleosides (or 2'-deoxyribonucleotides). The nucleosides or nucleotides may also be modified at the 3' position. The nucleosides or nucleotides may be specified as being either ribonucleosides (or ribonucleotides) or 2'-deoxyribonucleosides (or 2'-deoxyribonucleotides) on an individual basis or on an aggregate basis. When nucleic acid sequences are presented as a string of one-letter abbreviations, the sequences are presented in the 5' to 3' direction in accordance with common convention, and the phosphates are not indicated.

Definitions

[0055] In reference to the present invention, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise. Accordingly, the following terms are intended to have the following meanings.

[0056] "EC" number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0057] "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0058] "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0059] "Protein," "polypeptide," and "peptide" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids, as well as polymers comprising D- and L-amino acids, and mixtures of D- and L-amino acids.

[0060] "Amino acids" are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single letter codes.

[0061] As used herein, "polynucleotide," "oligonucleotide," and "nucleic acid'' are used interchangeably herein and refer to two or more nucleosides or nucleotides that are covalently linked together. The polynucleotide may be wholly comprised of ribonucleotides (i.e., RNA), wholly comprised of 2' deoxyribonucleotides (i.e., DNA), wholly comprised of other synthetic nucleotides or comprised of mixtures of synthetic, ribo- and/or 2' deoxyribonucleotides. The polynucleotides may also include modified nucleotides with substitutions, including 2' substitutions (e.g., 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, locked or constrained ethyl modifications, and others known to those skilled in the art). Nucleosides will be linked together via standard phosphodiester linkages or via one or more non-standard linkages, including but not limited to phosphorothioate linkages. The polynucleotide may be single-stranded or double-stranded or may include both single-stranded regions and double-stranded regions. Moreover, while a polynucleotide will typically be composed of the naturally occurring encoding nucleobases (i.e., adenine, guanine, uracil, thymine, and cytosine), it may include one or more modified and/or synthetic nucleobases, such as, for example, inosine, xanthine, hypoxanthine, etc. In some embodiments, such modified or synthetic nucleobases are nucleobases encoding amino-acid sequences. Nucleobases that are modified or synthetic may comprise any known or hypothetical or future discovered modification or structure that would be recognized by one of skill in the art as a modified or synthetic nucleobase. Similarly, the terms "polynucleotide," "oligonucleotide," and "nucleic acid'' are intended to comprise any modified or synthetic structure that is now known or discovered in the future that would be recognized by one of skill in the art as being or having the function of a "polynucleotide," "oligonucleotide," or "nucleic acid.'' An example of a modified or synthetic structure having the function of a "polynucleotide," "oligonucleotide," or "nucleic acid'' is PNA or peptide nucleic acid.

[0062] As used herein, "oligo acceptor substrate" and "acceptor substrate" and "growing oligo acceptor substrate strand" and "growing oligonucleotide chain" and "growing polynucleotide strand" are used interchangeably herein and refer to any oligo or nucleotide chain or similar moiety with an exposed 3'-OH or equivalent thereof that may be recognized by a wild-type TnT or polymerase or an engineered TnT or template-independent polymerase of the current disclosure as a substrate for nucleoside addition or synthesis. In some embodiments, the acceptor substrate may be single stranded. In yet other embodiments, the acceptor substrate may be double stranded or partially doubled stranded. In some embodiments, the acceptor substrate may comprise a nucleotide chain consisting of 1-10 nucleotides, 5-20 nucleotides, 15-50 nucleotides, 30-100 nucleotides, or greater than 100 nucleotides. In some embodiments, the acceptor substrate may comprise a

chemical moiety that is not a nucleotide chain but contains a free -OH capable of being recognized as a substrate by a wild-type or engineered TnT, referred to herein as a "3'-OH equivalent". Exemplary oligo acceptor substrates are provided in the Examples.

[0063] As used herein, "nucleoside triphosphate-3'-O-removable blocking group" and "nucleotide triphosphate-3'-O-removable blocking group" and "reversible terminator" and "NTP-3'-O-RBG" are used interchangeably herein and refer to a ribonucleoside triphosphate or a deoxyribonucleoside triphosphate or a synthetic or nucleoside triphosphate composed of an alternate or modified sugar with a removable blocking group attached at the 3' position of the sugar moiety. An NTP-3'-O-RBG may also include other modifications as described herein, including but not limited to modifications at the 2' position, modifications to the nucleobase, and modifications to the phosphates. A polynucleotide may also have a 3'-O-RBG, as is expected after reaction of an NTP-3'-O-RBG with an engineered TnT of the present disclosure and an oligo acceptor substrate.

[0064] As used herein, "oligo acceptor product" and "growing oligonucleotide chain" and "oligo acceptor extension product" are used interchangeably herein and refer to the product of a NTP-3'-O-RBG or other natural or modified NTP substrate and an oligo acceptor substrate, wherein a TnT or related polymerase has catalyzed the extension or addition of a nucleotide-3'-O-RBG or other natural or modified nucleotide substrate to an oligo acceptor substrate via reaction with one or more NTP-3'-O-RBGs or other natural or modified NTP substrates.

[0065] As used herein, "removable blocking group" and "blocking group" and "terminator group" and "reversible terminating group" and "inhibitor group" and related variations of these terms are used interchangeably herein and refer to a chemical group that would hinder addition of a second NTP-3'-O-RBG or other natural or modified NTP substrate to the 3' end of the growing oligo acceptor substrate strand prior to removal of the removable blocking from the first round of addition. In some embodiments, the NTP-3'-O-RBG or other natural or modified NTP substrate may comprise a removable blocking group selected from the group consisting of NTP-3'-O-NH_2, or NTP-3'-O-PO_3. In some embodiments, the NTP- 3'-O-RBG or other natural or modified NTP substrate may have a natural purine or pyrimidine base, such as adenine, guanine, cytosine, thymine, or uridine. In some embodiments, NTP- 3'-O-RBG or other natural or modified NTP substrates may have an unnatural base analog such as inosine, xanthine, hypoxanthine, or another base analog, as is known in the art. In some embodiments the blocking group may comprise or may additionally comprise a modification at the 2' position.

[0066] As used herein, "template-independent synthesis" refers to synthesis of an oligonucleotide or a polynucleotide without the use of template strand as a guide for synthesis of a complementary oligo or polynucleotide strand. Thus, template-independent synthesis refers to an iterative process, whereby, successive nucleotides are added to a growing oligo or nucleotide chain or acceptor substrate. Template-independent synthesis may be in a sequence defined manner or may be random, as is the case with the wild-type TnT in creating antigen receptor diversity. Processes for template-independent synthesis are further described herein.

[0067] "Coding sequence" refers to that portion of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0068] "Naturally-occurring" or "wild-type" refers to the form found in nature. For example, a naturally occurring or wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0069] As used herein, "recombinant," "engineered," and "non-naturally occurring" when used with reference to a cell, nucleic acid, or polypeptide, refer to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature. In some embodiments, the cell, nucleic acid, or polypeptide is identical to a naturally occurring cell, nucleic acid, or polypeptide, but is produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant cells expressing genes that are not found within the native (non-recombinant) form of the cell or expressed native genes that are otherwise expressed at a different level.

[0070] "Percentage of sequence identity" and "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides or polypeptides and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (Smith and Waterman, Adv. Appl. Math., 1981, 2:482), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, J. Mol. Biol., 1970, 48:443), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 1988, 85:2444), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms, which are described by Altschul et al. (See, Altschul et al., J. Mol. Biol., 1990, 215:403-410; and Altschul et al., Nucl. Acids Res., 1977, 3389-3402, respectively). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of

length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as, the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSum62 scoring matrix (See, Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA, 1989, 89:10915). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0071] "Reference sequence" refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity. In some embodiments, a "reference sequence" can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes in the primary sequence. For instance, a "reference sequence based on SEQ ID NO: 4 having at the residue corresponding to X14 a valine" or X14V refers to a reference sequence in which the corresponding residue at X14 in SEQ ID NO: 4, which is a tyrosine, has been changed to valine.

[0072] "Comparison window" refers to a conceptual segment of at least about 20 contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0073] As used herein, "substantial identity" refers to a polynucleotide or polypeptide sequence that has at least 80 percent sequence identity, at least 85 percent identity, at least between 89 to 95 percent sequence identity, or more usually, at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 residue positions, frequently over a window of at least 30-50 residues, wherein the percentage of sequence identity is calculated by comparing the reference sequence to a sequence that includes deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. In some specific embodiments applied to polypeptides, the term "substantial identity" means that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 89 percent sequence identity, at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). In some embodiments, residue positions that are not identical in sequences being compared differ by conservative amino acid substitutions.

[0074] "Corresponding to," "reference to," and "relative to" when used in the context of the numbering of a given amino acid or polynucleotide sequence refer to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered TnT, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0075] "Amino acid difference" or "residue difference" refers to a change in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as "Xn," where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a "residue difference at position X25 as compared to SEQ ID NO: 2" refers to a change of the amino acid residue at the polypeptide position corresponding to position 25 of SEQ ID NO:2. Thus, if the reference polypeptide of SEQ ID NO: 2 has a valine at position 25, then a "residue difference at position X25 as compared to SEQ ID NO:2" an amino acid substitution of any residue other than valine at the position of the polypeptide corresponding to position 25 of SEQ ID NO: 2. In most instances herein, the specific amino acid residue difference at a position is indicated as "XnY" where "Xn" specified the corresponding position as described above, and "Y" is the single letter identifier of the amino acid found in the engineered polypeptide (i.e., the different residue than in the reference polypeptide). In some embodiments, more than one amino acid can appear in a specified residue position (i.e., the alternative amino acids can be listed in the form XnY/Z, where Y and Z represent alternate amino acid residues). In some instances (e.g., in **Tables 5.2**, **6.2**, **7.2**, **8.2**, **9.2**, **10.2**, **11.2**, **12.2**, **13.2**, **14.2**, **15.2**, **16.2**, **17.2**, **18.2**, **19.2**, **20.2**, **21.2**, **22.2**, **23.2**, **24.2**, **25.2**, **26.2**, **27.2**, **28.2**, **29.2**, **30.2**, **35.2**, **36.2**, **37.2**, **38.2**, **39.2**, **40.2**, **41.2**, **42.2**, **43.2**, **44.2**, **45.2**, **46.2**, **47.2**, **48.2**, **49.2**, **50.2**, **51.2**, **52.2**, **53.2**, **54.2**, **56.2**, **57.2**, **58.2**, **59.2**, **60.2**, **61.2**, **62.2**, **63.2**, **64.2**, **65.2**, **66.2**, **67.2**, **68.2**, **69.2**, **70.2**, **71.2**, **72.2**, **73.2**, **74.2**, **75.2**, **76.2**, **77.2**, **78.2**, **79.2**, **80.2**, **81.2**, **82.2**, **83.2**, **84.2**, **85.2**, **86.2**, **87.2**, and **88.2**.) the

present invention also provides specific amino acid differences denoted by the conventional notation "AnB", where A is the single letter identifier of the residue in the reference sequence, "n" is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. Furthermore, in some instances, a polypeptide of the present invention can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where changes are made relative to the reference sequence. In some additional embodiments, the present invention provides engineered polypeptide sequences comprising both conservative and non-conservative amino acid substitutions.

[0076] As used herein, "conservative amino acid substitution" refers to a substitution of a residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain is substituted with another aliphatic amino acid (e.g., alanine, valine, leucine, and isoleucine); an amino acid with an hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain (e.g., serine and threonine); an amino acid having an aromatic side chain is substituted with another amino acid having an aromatic side chain (e.g., phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basic side chain (e.g., lysine and arginine); an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain (e.g., aspartic acid or glutamic acid); and/or a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively. Exemplary conservative substitutions are provided in **Table 1** below.

Table 1. Conservative Amino Acid Substitution Examples

Residue	Possible Conservative Substitutions
A, L, V, I	Other aliphatic (A, L, V, I) Other non-polar (A, L, V, I, G, M)
G, M	Other non-polar (A, L, V, I, G, M)
D, E	Other acidic (D, E)
K, R	Other basic (K, R)
N, Q, S, T	Other polar
H, Y, W, F	Other aromatic (H, Y, W, F)
C, P	None

[0077] "Non-conservative substitution" refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine), (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0078] "Deletion" refers to modification to the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or

more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the reference enzyme while retaining enzymatic activity and/or retaining the improved properties of an engineered TnT enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

[0079] "Insertion" refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. In some embodiments, the improved engineered TnT enzymes comprise insertions of one or more amino acids to the naturally occurring polypeptide as well as insertions of one or more amino acids to other improved TnT polypeptides. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0080] "Fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence. Fragments can be at least 14 amino acids long, at least 20 amino acids long, at least 50 amino acids long or longer, and up to 70%, 80%, 90%, 95%, 98%, and 99% of the full-length TnT polypeptide, for example the polypeptide of SEQ ID NO: 2 or an TnT provided in the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0081] "Functional fragment" and "biologically active fragment" are used interchangeably herein to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full-length engineered pyruvate oxidase of the present invention) and that retains substantially all of the activity of the full-length polypeptide.

[0082] "Isolated polypeptide" refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it, *e.g.*, protein, lipids, and polynucleotides. The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (*e.g.*, host cell or *in vitro* synthesis). The engineered TnT enzymes may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the engineered TnT enzyme can be an isolated polypeptide.

[0083] "Substantially pure polypeptide" refers to a composition in which the polypeptide species is the predominant species present (*i.e.*, on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition) and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure TnT composition will comprise about 60 % or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent

species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated engineered TnT polypeptide is a substantially pure polypeptide composition.

[0084] As used herein, "improved enzyme property" refers to at least one improved property of an enzyme. In some embodiments, the present invention provides engineered TnT polypeptides that exhibit an improvement in any enzyme property as compared to a reference TnT polypeptide and/or a wild-type TnT polypeptide, and/or another engineered TnT polypeptide. For the engineered TnT polypeptides described herein, the comparison is generally made to the wild-type enzyme from which the TnT is derived, although in some embodiments, the reference enzyme can be another improved engineered TnT. Thus, the level of "improvement" can be determined and compared between various TnT polypeptides, including wild-type, as well as engineered TnTs. Improved properties include, but are not limited, to such properties as enzymatic activity (which can be expressed in terms of percent conversion of the substrate), thermostability, solvent stability, pH activity profile, cofactor requirements, refractoriness to inhibitors (*e.g.*, substrate or product inhibition), activity at elevated temperatures, increased soluble expression, decreased by-product formation, increased specific activity on NTP-3'-O-RBG substrates, increased incorporation efficiency in extension of oligo acceptor substrates, and/or increased activity on various oligo acceptor substrates (including enantioselectivity).

[0085] "Increased enzymatic activity" refers to an improved property of the TnT polypeptides, which can be represented by an increase in specific activity (*e.g.*, product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (*e.g.,* percent conversion of starting amount of substrate to product in a specified time period using a specified amount of TnT) as compared to the reference TnT enzyme. Exemplary methods to determine enzyme activity are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m, V_{max} or k_{cat}, changes of which can lead to increased enzymatic activity. Improvements in enzyme activity can be from about 1.2 times the enzymatic activity of the corresponding wild-type enzyme, to as much as 2 times, 5 times, 10 times, 20 times, 25 times, 50 times or more enzymatic activity than the naturally occurring or another engineered TnT from which the TnT polypeptides were derived. TnT activity can be measured by any one of standard assays, such as by monitoring changes in properties of substrates, cofactors, or products. In some embodiments, the amount of products generated can be measured by Liquid Chromatography-Mass Spectrometry (LC-MS), HPLC, or other methods, as known in the art. Comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein. Generally, when lysates are compared, the numbers of cells and the amount of protein assayed are determined as well as use of identical expression systems and identical host cells to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0086] "Conversion" refers to the enzymatic conversion of the substrate(s) to the corresponding product(s). "Percent conversion" refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, the "enzymatic activity" or "activity" of a TnT polypeptide can be expressed as "percent conversion" of the substrate to the product.

[0087] "Thermostable" refers to a polypeptide that maintains similar activity (more than 60% to 80% for example) after exposure to elevated temperatures (*e.g.*, 40-80 °C) for a period of time (*e.g.*, 0.5-24 hrs) compared to the wild-type enzyme exposed to the same elevated temperature.

[0088] "Solvent stable" refers to a polypeptide that maintains similar activity (more than *e.g.*, 60% to 80%) after exposure to varying concentrations (*e.g.*, 5-99%) of solvent (ethanol, isopropyl alcohol, dimethylsulfoxide (DMSO), tetrahydrofuran, 2-methyltetrahydrofuran, acetone, toluene, butyl acetate, methyl tert-butyl ether, etc.) for a period of time (*e.g.*, 0.5-24 hrs) compared to the wild-type enzyme exposed to the same concentration of the same solvent.

[0089] "Thermo- and solvent stable" refers to a polypeptide that is both thermostable and solvent stable.

[0090] The term "stringent hybridization conditions" is used herein to refer to conditions under which nucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. In general, the stability of a hybrid is a function of ion strength, temperature, G/C content, and the presence of chaotropic agents. The T_m values for polynucleotides can be calculated using known methods for predicting melting temperatures (See e.g., Baldino et al., Meth. Enzymol., 1989, 168:761-777; Bolton et al., Proc. Natl. Acad. Sci. USA, 1962, 48:1390; Bresslauer et al., Proc. Natl. Acad. Sci. USA, 1986, 83:8893-8897; Freier et al., Proc. Natl. Acad. Sci. USA, 1986, 83:9373-9377; Kierzek et al., Biochem., 1986, 25:7840-7846; Rychlik et al., Nucl. Acids Res., 1990, 18:6409-6412 (erratum, Nucl. Acids Res., 1991, 19:698); Sambrook et al., *supra*); Suggs et al., 1981, *in* Developmental Biology Using Purified Genes, Brown et al., eds., pp. 683-693, Academic Press, Cambridge, MA (1981); and Wetmur, Crit. Rev. Biochem. Mol. Biol., 1991, 26:227-259). In some embodiments, the polynucleotide encodes the polypeptide disclosed herein and hybridizes under defined conditions, such as moderately stringent or highly stringent conditions, to the complement of a sequence encoding an engineered TnT enzyme of the present invention.

[0091] "Hybridization stringency" relates to hybridization conditions, such as washing conditions, in the hybridization of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term "moderately stringent hybridization" refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA, with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42 °C, followed by washing in 0.2×SSPE, 0.2% SDS, at 42 °C. "High stringency hybridization" refers generally to conditions that are about 10 °C or less from the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65 °C (*i.e.*, if a hybrid is not stable in 0.018M NaCl at 65 °C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42 °C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65 °C. Another high stringency condition is hybridizing

in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w:v) SDS at 65 °C and washing in 0.1x SSC containing 0.1% SDS at 65 °C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0092] "Heterologous" polynucleotide refers to any polynucleotide that is introduced into a host cell by laboratory techniques and includes polynucleotides that are removed from a host cell, subjected to laboratory manipulation, and then reintroduced into a host cell.

[0093] "Codon optimized" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. Although the genetic code is degenerate in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the TnT enzymes may be codon optimized for optimal production from the host organism selected for expression.

[0094] As used herein, "preferred, optimal, high codon usage bias codons" refers interchangeably to codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid. The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. Codons whose frequency increases with the level of gene expression are typically optimal codons for expression. A variety of methods are known for determining the codon frequency (e.g., codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariate analysis, for example, using cluster analysis or correspondence analysis, and the effective number of codons used in a gene (See e.g., GCG CodonPreference, Genetics Computer Group Wisconsin Package; CodonW, Peden, University of Nottingham; McInerney, Bioinform., 1998, 14:372-73; Stenico et al., Nucl. Acids Res., 1994, 222437-46; Wright, Gene, 1999, 87:23-29). Codon usage tables are available for many different organisms (See e.g., Wada et al., Nucl. Acids Res., 1992, 20:2111-2118; Nakamura et al., Nucl. Acids Res., 2000, 28:292; Duret, et al., supra; Henaut and Danchin, *in* Escherichia coli and Salmonella, Neidhardt, et al. (eds.), ASM Press, Washington D.C., p. 2047-2066 (1996)). The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (e.g., complete protein coding sequences-CDS), expressed sequence tags (ESTS), or predicted coding regions of genomic sequences (See e.g., Mount, Bioinformatics: Sequence and Genome Analysis, Chapter 8, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Uberbacher, Meth. Enzymol., 1996, 266:259-281; and Tiwari et al., Comput. Appl. Biosci., 1997, 13:263-270).

[0095] "Control sequence" is defined herein to include all components, which are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present invention. Each control sequence may

be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

[0096] "Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed (*i.e.*, in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide and/or polypeptide of interest.

[0097] "Promoter sequence" refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0098] "Suitable reaction conditions" refer to those conditions in the biocatalytic reaction solution (*e.g.*, ranges of enzyme loading, substrate loading, cofactor loading, temperature, pH, buffers, co-solvents, etc.) under which a TnT polypeptide of the present invention is capable of converting one or more substrate compounds to a product compound (*e.g.*, addition of a nucleotide-3'-O-RBG or other natural or modified nucleotide substrate to an oligo acceptor substrate via reaction with NTP-3'-O-RBG or other natural or modified NTP substrate). Exemplary "suitable reaction conditions" are provided in the present invention and illustrated by the Examples.

[0099] "Composition" refers to a mixture or combination of one or more substances, wherein each substance or component of the composition retains its individual properties. As used herein, a biocatalytic composition refers to a combination of one or more substances useful for biocatalysis.

[0100] "Loading", such as in "compound loading" or "enzyme loading" or "cofactor loading" refers to the concentration or amount of a component in a reaction mixture at the start of the reaction.

[0101] "Substrate" in the context of a biocatalyst mediated process refers to the compound or molecule acted on by the biocatalyst. For example, a TnT biocatalyst used in the synthesis processes disclosed herein acts on an NTP-3'-O-RBG substrate or other natural or modified NTP substrate and an oligo acceptor substrate.

[0102] "Product" in the context of a biocatalyst mediated process refers to the compound or molecule resulting from the action of the biocatalyst. For example, an exemplary product for a TnT biocatalyst used in a process disclosed herein is an oligo acceptor extension product, as depicted in Schemes 1 and 2.

[0103] "Alkyl" refers to saturated hydrocarbon groups of from 1 to 18 carbon atoms inclusively, either straight chained or branched, more preferably from 1 to 8 carbon atoms inclusively, and most preferably 1 to 6 carbon atoms inclusively. An alkyl with a specified number of carbon atoms is denoted in parenthesis (e.g., $(C_1\text{-}C_6)$alkyl refers to an alkyl of 1 to 6 carbon atoms).

[0104] "Alkenyl" refers to hydrocarbon groups of from 2 to 12 carbon atoms inclusively, either straight or branched containing at least one double bond but optionally containing more than one double bond.

[0105] "Alkynyl" refers to hydrocarbon groups of from 2 to 12 carbon atoms inclusively, either straight or branched containing at least one triple bond but optionally containing more than one triple bond, and additionally optionally containing one or more double bonded moieties.

[0106] "Heteroalkyl, "heteroalkenyl," and heteroalkynyl," refer respectively, to alkyl, alkenyl and alkynyl as defined herein in which one or more of the carbon atoms are each independently replaced with the same or different heteroatoms or heteroatomic groups. Heteroatoms and/or heteroatomic groups which can replace the carbon atoms include, but are not limited to -O-, -S-, -S-O-, -NR^g-, -PH-, -S(O)-, -S(O)2-, -S(O) NR^g-, -$S(O)_2NR^g$, and the like, including combinations thereof, where each R^g is independently selected from hydrogen, alkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

[0107] "Amino" refers to the group -NH_2. Substituted amino refers to the group –NHR^h, NR^hR^h, and $NR^hR^hR^h$, where each R^h is independently selected from substituted or unsubstituted alkyl, cycloalkyl, cycloheteroalkyl, alkoxy, aryl, heteroaryl, heteroarylalkyl, acyl, alkoxycarbonyl, sulfanyl, sulfinyl, sulfonyl, and the like. Typical amino groups include, but are limited to, dimethylamino, diethylamino, trimethylammonium, triethylammonium, methylysulfonylamino, furanyl-oxy-sulfamino, and the like.

[0108] "Aminoalkyl" refers to an alkyl group in which one or more of the hydrogen atoms are replaced with one or more amino groups, including substituted amino groups.

[0109] "Aminocarbonyl" refers to -$C(O)NH_2$. Substituted aminocarbonyl refers to –$C(O)NR^hR^h$, where the amino group NR^hR^h is as defined herein.

[0110] "Oxy" refers to a divalent group -O-, which may have various substituents to form different oxy groups, including ethers and esters.

[0111] "Alkoxy" or "alkyloxy" are used interchangeably herein to refer to the group –OR^z, wherein R^z is an alkyl group, including optionally substituted alkyl groups.

[0112] "Carboxy" refers to -COOH.

[0113] "Carbonyl" refers to -C(O)-, which may have a variety of substituents to form different carbonyl groups including acids, acid halides, aldehydes, amides, esters, and ketones.

[0114] "Carboxyalkyl" refers to an alkyl in which one or more of the hydrogen atoms are replaced with one or more carboxy groups.

[0115] "Aminocarbonylalkyl" refers to an alkyl substituted with an aminocarbonyl group, as defined herein.

[0116] "Halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

[0117] "Haloalkyl" refers to an alkyl group in which one or more of the hydrogen atoms are replaced with a halogen. Thus, the term "haloalkyl" is meant to include monohaloalkyls, dihaloalkyls, trihaloalkyls, etc. up to perhaloalkyls. For example, the expression "(C_1 - C_2) haloalkyl" includes 1-fluoromethyl, difluoromethyl, trifluoromethyl, 1-fluoroethyl, 1,1-difluoroethyl, 1,2-difluoroethyl, 1,1,1 trifluoroethyl, perfluoroethyl, etc.

[0118] "Hydroxy" refers to -OH.

[0119] "Hydroxyalkyl" refers to an alkyl group in which in which one or more of the hydrogen atoms are replaced with one or more hydroxy groups.

[0120] "Thiol" or "sulfanyl" refers to –SH. Substituted thiol or sulfanyl refers to –S-R^h, where R^h is an alkyl, aryl, or other suitable substituent.

[0121] "Sulfonyl" refers to –SO_2-. Substituted sulfonyl refers to –SO_2-R^h, where R^h is an alkyl, aryl, or other suitable substituent.

[0122] "Alkylsulfonyl" refers to –SO_2-R^z, where R^z is an alkyl, which can be optionally substituted. Typical alkylsulfonyl groups include, but are not limited to, methylsulfonyl, ethylsulfonyl, n-propylsulfonyl, and the like.

[0123] "Phosphate" as used herein refers to a functional group comprised of an orthophosphate ion (phosphorous atom covalently linked to four oxygen atoms). The orthophosphate ion is commonly found with one or more hydrogen atoms or organic groups.

[0124] "Phosphorylated" as used herein refers to the addition or presence of one of more phosphoryl groups (phosphorous atom covalently linked to the three oxygen atoms).

[0125] "Optionally substituted" as used herein with respect to the foregoing chemical groups means that positions of the chemical group occupied by hydrogen can be substituted with another atom (unless otherwise specified) exemplified by, but not limited to carbon, oxygen, nitrogen, or sulfur, or a chemical group, exemplified by, but not limited to, hydroxy, oxo, nitro, methoxy, ethoxy, alkoxy, substituted alkoxy, trifluoromethoxy, haloalkoxy, fluoro, chloro, bromo, iodo, halo, methyl, ethyl, propyl, butyl, alkyl, alkenyl, alkynyl, substituted alkyl, trifluoromethyl, haloalkyl, hydroxyalkyl, alkoxyalkyl, thio, alkylthio, acyl, carboxy, alkoxycarbonyl, carboxamido, substituted carboxamido, alkylsulfonyl, alkylsulfinyl, alkylsulfonylamino, sulfonamido, substituted sulfonamido, cyano, amino, substituted amino, alkylamino, dialkylamino, aminoalkyl, acylamino, amidino, amidoximo, hydroxamoyl, phenyl, aryl, substituted aryl, aryloxy, arylalkyl, arylalkenyl, arylalkynyl, pyridyl, imidazolyl, heteroaryl, substituted heteroaryl, heteroaryloxy, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloalkyl, cycloalkenyl, cycloalkylalkyl, substituted cycloalkyl, cycloalkyloxy, pyrrolidinyl, piperidinyl, morpholino, heterocycle, (heterocycle)oxy, and (heterocycle)alkyl; where preferred heteroatoms are oxygen, nitrogen, and sulfur. Additionally, where open valences exist on these substitute chemical groups they can be further substituted with alkyl, cycloalkyl, aryl, heteroaryl, and/or heterocycle groups, that where these open valences exist on carbon they can be further substituted by halogen and by oxygen-, nitrogen-, or sulfur-bonded substituents, and where multiple such open valences exist, these groups can be joined to form a ring, either by direct formation of a bond or by formation of bonds to a new heteroatom, preferably oxygen, nitrogen, or sulfur. It is further contemplated that the above substitutions can be made provided that replacing the hydrogen with the substituent does not introduce unacceptable instability to the molecules of the present invention and is otherwise chemically reasonable. One of ordinary skill in the art would understand that with respect to any chemical group described as optionally substituted, only sterically practical and/or synthetically

feasible chemical groups are meant to be included. "Optionally substituted" as used herein refers to all subsequent modifiers in a term or series of chemical groups. For example, in the term "optionally substituted arylalkyl," the "alkyl" portion and the "aryl" portion of the molecule may or may not be substituted, and for the series "optionally substituted alkyl, cycloalkyl, aryl and heteroaryl," the alkyl, cycloalkyl, aryl, and heteroaryl groups, independently of the others, may or may not be substituted.

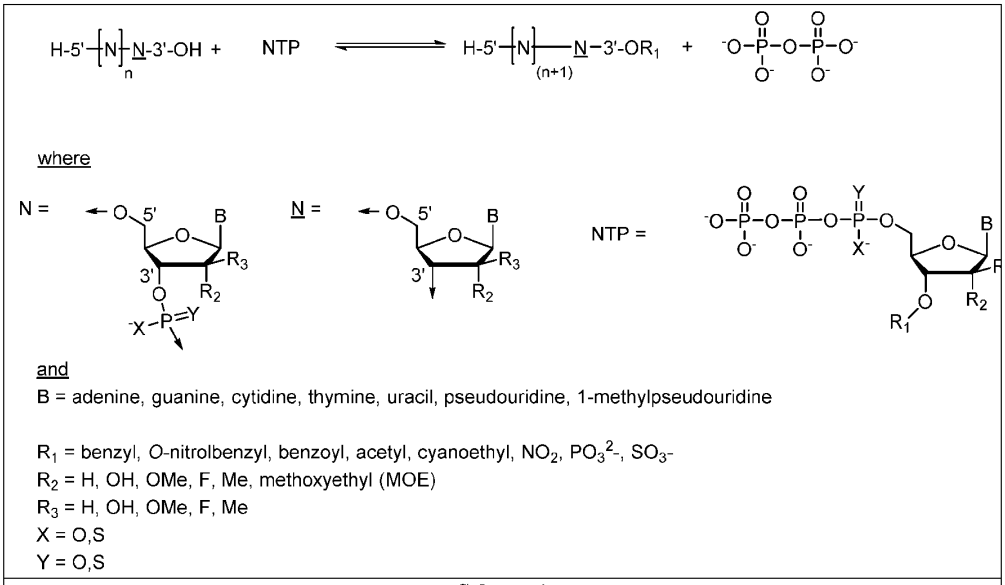
[0126] "Reaction" as used herein refers to a process in which one or more substances or compounds or substrates is converted into one or more different substances, compounds, or processes.

Template-Independent Synthesis by Engineered TnTs

[0127] New methods of efficiently synthesizing high purity strands of DNA, RNA, and other polynucleotides are necessary to overcome the limitations of existing phosphoramidite chemical synthesis methods in order to enable a range of emerging and existing synthetic biology applications.

[0128] The present invention provides novel terminal nucleotidyl transferases that have improved activity in the template-independent synthesis of polynucleotides using 5'-nucleoside triphosphates ("NTPs") modified with a 3'-O-removable blocking group (NTP-3'-O-RBG) or other natural or modified NTP substrates. The TnTs of the present disclosure have improved thermostability, activity at elevated temperatures, increased soluble expression or isolated protein yield, decreased by-product formation, increased affinity for NTP-3'-O-RBG and other natural or modified NTP substrates, increased affinity for oligo acceptor substrates, increased activity or specific activity on NTP-3'-O-RBG and other natural or modified NTP substrates, and/or increased activity or specific activity on various oligo acceptor substrates as compared to a wild-type TnT or other TnTs or template-independent polymerases known to those of skill in the art. The engineered polypeptides of the present disclosure are variants of the engineered TnT enzyme of SEQ ID NO: 2 (PCT/US2023/076667), which is an engineered variant of a predicted splice variant of the wild-type gene from *Monodelphis domestica*. These engineered TnTs are capable of template-independent synthesis of oligonucleotides and polynucleotides.

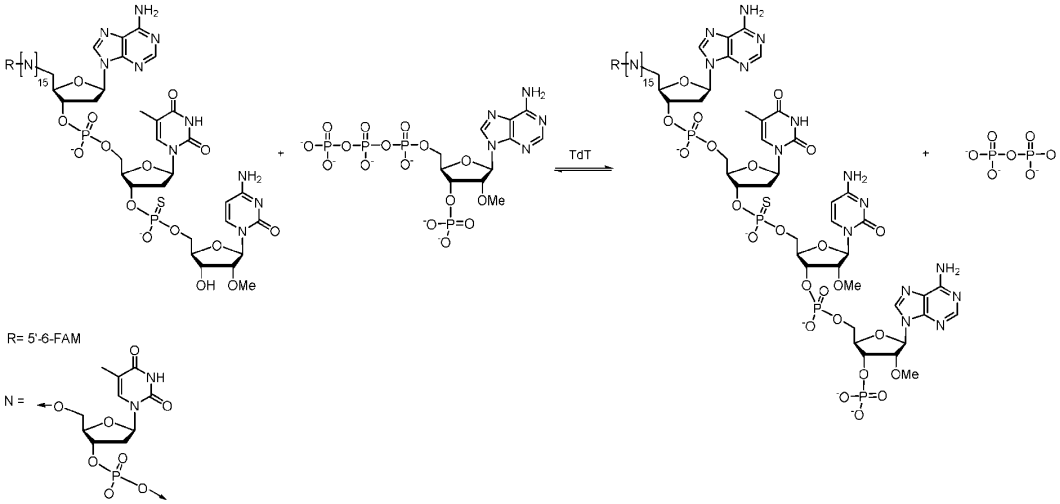
[0129] Template-independent synthesis of a defined polynucleotide sequence using an engineered TnT is a multistep process. In one embodiment, an oligo acceptor substrate with a 3'-OH allows addition of a defined modified NTP substrate (in this example, an NTP-3'-O-RBG) by an engineered TnT, as depicted in **Scheme 1**, below.



Scheme 1

[0130] After reaction of the NTP-3'-O-RBG with the 3'-OH of oligo acceptor substrate or the growing polynucleotide chain, the TnT is blocked from further reaction by the 3'-O-RBG. The RBG is then removed, exposing the 3'-OH and allowing another round of addition. After each round of addition, the blocking group of the nucleotide-3'-O-RBG or natural or modified nucleotide from the previous round is removed and a new NTP-3'-O-RBG or natural or modified NTP substrate is added to sequentially and efficiently create a defined polynucleotide sequence by addition at the 3'-OH end of the polynucleotide or oligo acceptor substrate without a complimentary strand or templating primer sequence. After synthesis of the defined polynucleotide is complete, the oligonucleotide chain may be cleaved or released from the oligo acceptor substrate.

[0131] A variety of oligo acceptor substrates and NTP-3'-O-RBG or natural or modified NTP substrates may be used in this process, as may be envisioned by one of skill in the art. An example of one reaction is detailed in Scheme 2, below. Scheme 2 depicts the TnT catalyzed reaction of 5'-6-FAM-$[N]_{15}$AT*mC and 3'-phos-mATP, while other examples of suitable oligo acceptor substrate and NTP-3'-O-RBG or natural or modified NTP pairs are described in the Examples. These examples are non-limiting.



Scheme 2

[0132] Occasionally, undesired synthesis products are created by the TnT during the addition step. This includes incorporation of NTPs that have lost their blocking group, addition of more than one NTP, or the excision or pyrophosphorolysis of the TnT on the growing polynucleotide chain.

[0133] In some embodiments, one or more additional quality control steps are used, such as adding an exonuclease prior to removing the blocking group and initiating a new round of synthesis. In some embodiments, a phosphatase, such as a pyrophosphatase, is used to breakdown inorganic phosphate and push the reversible TnT reaction toward synthesis.

[0134] As described further herein, the engineered TnT polypeptides of the current disclosure exhibit one of more improved properties in the template-independent polynucleotide synthesis process depicted in Schemes 1 and 2.

[0135] In some embodiments, the present invention provides an engineered TnT polypeptide comprising an amino acid sequence having at least 60% sequence identity to an amino acid reference sequence of SEQ ID NO: 2 and further comprising one or more amino acid residue differences as compared to the reference amino acid sequence, wherein the engineered TnT polypeptide has improved thermostability, increased activity at elevated temperatures, increased soluble expression or isolated protein yield, decreased by-product formation, increased specific activity on NTP-3'-O-RBG or natural or modified NTP substrates, and/or increased activity on various oligo acceptor substrates as compared to a wild-type TnT or other TnTs or template-independent polymerases known to those of skill in the art.

[0136] In particular, the engineered TnTs polypeptides of the present disclosure have been engineered for efficient synthesis of polynucleotides having a defined sequence using NTP-3'-O-RBG or natural or modified NTP substrates in the process described above.

[0137] A variety of suitable reaction conditions are known to those skilled in the art, as detailed below and in the Examples.

Engineered Terminal Nucleotidyl Transferase (TnT) Polypeptides

[0138] The present invention provides engineered terminal nucleotidyl transferase (TnT) polypeptides useful in template-independent polynucleotide synthesis using an NTP-3'-O-RBG or natural or modified NTP substrate, as well as compositions and methods of utilizing these engineered polypeptides in template-independent oligonucleotide synthesis.

[0139] The present invention provides TnT polypeptides, polynucleotides encoding the polypeptides, methods of preparing the polypeptides, and methods for using the polypeptides. Where the description relates to polypeptides, it is to be understood that it can describe the polynucleotides encoding the polypeptides.

[0140] Suitable reaction conditions under which the above-described improved properties of the engineered polypeptides carry out the desired reaction can be determined with respect to concentrations or amounts of polypeptide, substrate, co-substrate, buffer, solvent, pH, conditions including temperature and reaction time, and/or conditions with the polypeptide immobilized on a solid support, as further described below and in the Examples.

[0141] In some embodiments, exemplary engineered TnTs comprise an amino acid sequence that has one or more residue differences as compared to SEQ ID NO: 2 at the residue positions indicated in **Tables 5.2, 6.2, 7.2, 8.2, 9.2, 10.2, 11.2, 12.2, 13.2, 14.2, 15.2, 16.2, 17.2, 18.2, 19.2, 20.2, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 35.2, 36.2, 37.2, 38.2, 39.2, 40.2, 41.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 49.2, 50.2, 51.2, 52.2, 53.2, 54.2, 56.2, 57.2, 58.2, 59.2, 60.2, 61.2, 62.2, 63.2, 64.2, 65.2, 66.2, 67.2, 68.2, 69.2, 70.2, 71.2, 72.2, 73.2, 74.2, 75.2, 76.2, 77.2, 78.2, 79.2, 80.2, 81.2, 82.2, 83.2, 84.2, 85.2, 86.2, 87.2,** and **88.2**.

[0142] The structure and function information for the exemplary engineered polypeptides of the present invention are based on the conversion of an oligo acceptor substrate and a NTP -3'-O-RBG or a dideoxy NTP (e.g., a 2',3'-dideoxy NTP), the results of which are shown below in **Tables 5.2, 6.2, 7.2, 8.2, 9.2, 10.2, 11.2, 12.2, 13.2, 14.2, 15.2, 16.2, 17.2, 18.2, 19.2, 20.2, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 35.2, 36.2, 37.2, 38.2, 39.2, 40.2, 41.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 49.2, 50.2, 51.2, 52.2, 53.2, 54.2, 56.2, 57.2, 58.2, 59.2, 60.2, 61.2, 62.2, 63.2, 64.2, 65.2, 66.2, 67.2, 68.2, 69.2, 70.2, 71.2, 72.2, 73.2, 74.2, 75.2, 76.2, 77.2, 78.2, 79.2, 80.2, 81.2, 82.2, 83.2, 84.2, 85.2, 86.2, 87.2,** and **88.2**, as further described in the Examples. The odd numbered sequence identifiers (i.e., SEQ ID NOs) in these Tables refer to the nucleotide sequence encoding the amino acid sequence provided by the even numbered SEQ ID NOs in these Tables. Exemplary sequences are provided in the electronic Sequence Listing file accompanying this disclosure, which is hereby incorporated by reference herein. The amino acid residue differences are based on comparison to the reference sequence of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, as indicated. It is to be understood that the amino acid residue differences can also be made in comparison to the reference sequence without the histidine-tag, e.g., a reference sequence corresponding to amino acid residues 12 to the

carboxy terminus of an even-numbered SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0143] Terminal deoxynucleotidyl transferase, a member of the Pol X family, has been identified in many species. Members of the diverse Pol X family are known to share certain residues, which are conserved across family members. TdT also has a high level of conservation across species for residues thought to be involved in binding divalent metal ions, ternary complex formation, and binding dNTP and DNA ligands (Domínguez et al. (2000). EMBO, 19(7), 1731–1742.) Additionally, TdTs are known to have splice variants which are N-terminal truncations, lacking a BRCT domain.

[0144] Other template-independent polymerases (including, but not limited to Poly(N) polymerases, Polμ polymerases, Polβ polymerases, Polλ polymerases, terminal urildylytransferases, and Polθ polymerases, and others) are also known in the art and may be used to practice the invention (see for example, U.S. Pat. 10059929, U.S. Pat. 10,760,063, U.S. Pat. 10,774,316, U.S. Pat. 11,390,858, U.S. Pat. 10,745,727, PCT/GB2020/050247, US20210164008A1, US20210407509, WO2016GB50301, WO17216472, WO18215803, WO20072715, WO20077227, WO21122539, WO2022029427, and WO21116270, from Molecular Assemblies, Nuclera, DNA Script, and others, each of which is specifically incorporated herein).

[0145] Similarly, other polymerases are known to be capable of template-independent synthesis (including but not limited to reverse transcriptases) and may be used to practice the invention.

[0146] As used herein, the term terminal nucleotidyl transferase (TnT) is used to distinguish an engineered enzyme with activity on a variety of nucleoside triphosphates, including ribonucleoside triphosphates with 3' modifications or with 2' modifications or with 2' and 3' modifications, from a wild-type TdT enzyme having wild-type TdT activity.

[0147] The engineered TnT enzyme of SEQ ID NO: 2 (PCT/US2023/076667, published as WO2024081770), which is an engineered variant of a predicted splice variant of the wild-type gene from *Monodelphis domestica*, was selected for evolution. The TnT polypeptides of the present disclosure are engineered variants of SEQ ID NO: 2 with a N-terminal 6-histidine tag.

[0148] The polypeptides of the present disclosure have residue differences that result in improved properties necessary to develop an efficient TnT enzyme, capable of template-independent synthesis of polynucleotides, particularly polynucleotides having a defined sequence. Various residue differences, at both conserved and non-conserved positions, have been discovered to be related to improvements in various enzymes properties, including improved thermostability, increased activity at elevated temperatures, increased soluble expression or isolated protein yield, decreased by-product formation, increased specific activity on NTP-3'-O-RBG or natural or modified NTP substrates, increased incorporation efficiency in extension of oligo acceptor substrates, and/or increased activity on various oligo acceptor substrates as compared to a wild-type TnT or other TnTs or template-independent polymerases known to those of skill in the art. In some embodiments, the engineered TnT polypeptides exhibit increased incorporation efficiency in extension of an oligo acceptor substrate by addition of an NTP or NQP of greater than 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%,

93%, 94%, 95%, 96%, 97%, 98%, or 99%. Exemplary incorporation efficiency of engineered TnTs is described in the Examples.

[0149] The polypeptides of the present disclosure are capable of incorporation of various NTP-3'-O-RBG or natural or modified NTP substrates using an optionally modified oligo acceptor substrate with a length of three to seven nucleotides, as provided in Examples 31 and 32. In some embodiments, the incorporation efficiency of the TnT polypeptides described herein is enhanced by addition of a phosphate to the 5' end of an optionally modified oligo acceptor substrate with a length of three to seven nucleotides.

[0150] The activity of each engineered TnT relative to the reference polypeptide of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, was determined as conversion of the substrates described in the Examples herein. In some embodiments, a shake flask purified enzyme (SFP) is used to assess the properties of the engineered TnTs, the results of which are provided in the Examples.

[0151] In some embodiments, the specific enzyme properties are associated with the residues differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002 at the residue positions indicated herein. In some embodiments, residue differences affecting polypeptide expression can be used to increase expression of the engineered TnTs.

[0152] In light of the guidance provided herein, it is further contemplated that any of the exemplary engineered polypeptides comprising the sequences of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002 find use as the starting amino acid sequence for synthesizing other TnT polypeptides, for example by subsequent rounds of evolution that incorporate new combinations of various amino acid differences from other polypeptides in **Tables 5.2, 6.2, 7.2, 8.2, 9.2, 10.2, 11.2, 12.2, 13.2, 14.2, 15.2, 16.2, 17.2, 18.2, 19.2, 20.2, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 35.2, 36.2, 37.2, 38.2, 39.2, 40.2, 41.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 49.2, 50.2, 51.2, 52.2, 53.2, 54.2, 56.2, 57.2, 58.2, 59.2, 60.2, 61.2, 62.2, 63.2, 64.2, 65.2, 66.2, 67.2, 68.2, 69.2, 70.2, 71.2, 72.2, 73.2, 74.2, 75.2, 76.2, 77.2, 78.2, 79.2, 80.2, 81.2, 82.2, 83.2, 84.2, 85.2, 86.2, 87.2,** and **88.2**, and other residue positions described herein. Further improvements may be generated by including amino acid differences at residue positions that had been maintained as unchanged throughout earlier rounds of evolution.

[0153] In one aspect, the engineered terminal nucleotidyl transferase, or a functional fragment thereof, comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of an even-numbered SEQ ID NO. of SEQ ID NOs: 2, 4-1056, 1130-1880, and 1928-3008, or to a reference sequence corresponding to SEQ ID NO: 2, 4-1056, 1130-1880, and 1928-3008, wherein the amino acid sequence

comprises one or more amino differences relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0154] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, wherein the amino acid sequence comprises one or more amino differences relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0155] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, or 526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy

terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0156] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue 22P, 27P, 34Y, 41T, 42T, 43-/T, 45T, 52G, 60H, R92C, 105G, 133E, 152H, 160A/D/T/W, 161A/G/L/Q/T, 162P, 164Q, 169L, 173G/H/P, 174L/V/W, 175E/H/L/P/R/S/T/V, 176H, 177C/K, 179C/K/L/R/V, 182I/V, 183G/R/S/T/V, 184K/R, 186G/L/M/R/T/V, 187L/T/V/Y, 190A/F/G/H/L/M/N/Q/R/T/V, 191F/W/Y, 192A/H/S, 193A/G/I/Q/R/S/T/W, 194L/M, 195F/Q/R/T/W, 196D/H/K/L/Q/R/S/V, 197I/L/M/Q/R, 198E/H/P/Q/R/S/T, 199E/F/V, 200Q, 201D/E/P/Q/R/S, 202H/I/M/S/V, 203F/L/M/Q, 204M/V, 205L 208A/D/E/G/M/N/Q/T, 212R, 213G, 218I, 219D/Q/S, 221I/Q/V, 222A/N, 223H/L/T, 224L/V, 227G/S, 229F/G/L/S, 230C/L/V, 231C/M/N/T, 232E/G, 233I/P, 234T, 235N/R, 236R/S, 237L, 238F/I/L/V, 239G/L/R/S, 241L, 242L, 244G/I/R/S, 246L/V, 249L/M/Q, 250G, 251A/E/G/M/R/S/W, 252E/H/L/M/R/S, 254I/N/Q/R, 259F, 260K/R/S/T, 261G/R/T/V, 262L/M, 263A/D/K/R/S/T/W, 264L/R/S, 267G/S, 268L, 272R, 273E/G/I/K/L/M//N/Q/R, 274L/N/P/R/S/T/V, 275I/L/R/S, 277E/S/T, 278A/D/L/R/S, 280W, 281A/C/G/H/L/S, 282N, 288D, 290L, 291K/L, 292A, 293R, 294G/T, 295A/D/N/T, 296M/V, 297A/G/L/M/P/Q/S/T/V/W/Y, 298I, 299A/G/H/N/P/R/S, 300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, 301A/F/V, 302S, 303E/R/T, 304L, 307D/E/K/M/N/S, 308H/L, 309H, 310D/S, 312M, 313H/Q/R/S/T, 314A/E/I/S/T, 315K/R, 316L, 317R, 318D/I/K/L/P/Q/S/T, 319G/L/M/P/Q/T/V/W, 321R/T, 322A/E, 323T, 324A/T, 325G/S/V/Y, 328A/I, 329H/L, 333N/S, 334S, 335V, 336C/T, 337S, 339I/S/T/V, 340L/M/T/V, 341L/T, 342S/T, 343L/M/R/T/V, 347A/S, 352A/E/L/M/N/S/T/V, 353E/K/L/R/T/V, 354G, 357I, 359A/C/F/V, 360V, 361L/V, 362A/M, 363G/P/T, 364S, 366D/F/H/P, 367N/Q/R/S/V, 368L/N/R/S, 370E/F/L/R, 371G/R, 376A/E/G/H/R/S/T/V, 377R, 378M, 379L/T, 380A/K/L/S/V, 381S/Y, 382L/M, 383L/Q, 384A/G/S/T, 385V, 387T/V, 388V, 389A/V, 390I/L/M/S/T, 392E/H/N/Q/R/S, 393A/G/I/L/M/S/T/Y, 394K/N/S, 395E/F/I/L/M/Q/S/T, 396T, 397E/F/G/H/I/K/L/M/R/S/T/V/W/Y, 398L/M, 402A/D/K/M/P/S/T, 403E/F/I/P, 404A/E/G/I/L/M/Q/R/S/T/V, 405A/G/L/P/Q/T/V/Y, 406A/D/E/H/M/N/P/R/T, 407A/D/G/L/N/P/Q/S/V/Y, 408D/G/L/N/S/V, 409E/G/K/N/P/Q/R/S/V/Y, 410V, 411E/H/K/R/T/V, 412M/N/P/Q/S/V, 413S/T, 414M/Q/R/V, 415G/P/S, 416A/G/H, 417A/G/S, 418C/S/T, 419A/I/M, 420C/L/V, 424P, 425R, 426G, 427A/M, 428A/L/T, 429A/S, 430G/Q/S/W, 431G, 432I/V, 433A/P/T/V, 434G/L/P, 435F/L/N/T/V, 436C/G/H/L/S/T, 437A/H/P/S, 438P/V, 439C/L/P, 440D/M, 441E/T, 442E/L/M/R/V, 443A/D/P/V, 444G, 446D/G/H/S/T, 448R, 450V, 451R, 456M, 457T, 458K, 459L, 460A/D/H/L/Q/R/S/V, 461A/V, 462H/L/M/Y, 463P/S, 465G, 466I/L, 467M, 468H/I/M, 474H/I/L/V, 477A/E/G/I/L/Q/S/T/W, 479M/T/V, 480K/M/W, 481H, 484A/L/W/Y, 485I/L/V, 487R, 490A/I/L/V, 492A/C/G/H/M/R/S/Y, 493R, 494L/M/Q/R/T/V/Y, 495A/G/L/T, 497L, 499L/N/R/S, 500A/N, 501K/L, 502M, 503L/R/V, 504P/S/T, 506A/K/L, 507G, 508C/D/L/Q, 513V, 515G, 517I, 518S, 522A/F/M/V, 523A/D/H, 525G/H/I/R/T/V/W, or 526E/G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to

amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

[0157] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue F22P, Q27P, D34Y, I41T, K42T, F43-/T, E45T, E52G, R60H, R92C, E105G, K133E, R152H, M160A/D/T/W, K161A/G/L/Q/T, T162P, S164Q, Q169L, T173G/H/P, L174L/V/W, N175E/H/L/P/R/S/T/V, N176H, R177C/K, Q179C/K/L/R/V, T182I/V, D183G/R/S/T/V, S184K/R, E186G/L/M/R/T/V, I187L/T/V/Y, E190A/F/G/H/L/M/N/Q/R/T/V, N191F/W/Y, Y192A/H/S, V193A/G/I/Q/R/S/T/W, F194L/M, K195F/Q/R/T/W, G196D/H/K/L/Q/R/S/V, N197I/L/M/Q/R, D198E/H/P/Q/R/S/T, D199E/F/V, K200Q, A201D/E/P/Q/R/S, L202H/I/M/S/V, E203F/L/M/Q, F204M/V, R205L I208A/D/E/G/M/N/Q/T, K212R, S213G, V218I, R219D/Q/S, L221I/Q/V, K222A/N, D223H/L/T, T224L/V, L227G/S, W229F/G/L/S, I230C/L/V, S231C/M/N/T, D232E/G, E233I/P, V234T, K235N/R, G236R/S, I237L, M238F/I/L/V, E239G/L/R/S, I241L, I242L, V244G/I/R/S, E246L/V, E249L/M/Q, V250G, Q251A/E/G/M/R/S/W, A252E/H/L/M/R/S, L254I/N/Q/R, Y259F, Q260K/R/S/T, S261G/R/T/V, F262L/M, H263A/D/K/R/S/T/W, A264L/R/S, R267G/S, V268L, G272R, V273E/G/I/K/L/M//N/Q/R, G274L/N/P/R/S/T/V, T275I/L/R/S, D277E/S/T, K278A/D/L/R/S, Y280W, Q281A/C/G/H/L/S, M282N, E288D, V290L, R291K/L, S292A, S293R, K294G/T, T295A/D/N/T, L296M/V, K297A/G/L/M/P/Q/S/T/V/W/Y, L298I, T299A/G/H/N/P/R/S, T300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, W301A/F/V, A302S, K303E/R/T, V304L, T307D/E/K/M/N/S, Y308H/L, Y309H, E310D/S, L312M, A313H/Q/R/S/T, R314A/E/I/S/T, G315K/R, V316L, T317R, R318D/I/K/L/P/Q/S/T, , A319G/L/M/P/Q/T/V/W, A321R/T, D322A/E, A323T, I324A/T, W325G/S/V/Y, V328A/I, Q329H/L, A333N/S, T334S, F335V, D336C/T, P337S, A339I/S/T/V, I340L/M/T/V, V341L/T, E342S/T, I343L/M/R/T/V, Q347A/S, P352A/E/L/M/N/S/T/V, Q353E/K/L/R/T/V, S354G, V357I, L359A/C/F/V, L360V, I361L/V, S362A/M, S363G/P/T, P364S, A366D/F/H/P, E367N/Q/R/S/V, K368L/N/R/S, Q370E/F/L/R, E371G/R, Q376A/E/G/H/R/S/T/V, K377R, V378M, M379L/T, R380A/K/L/S/V, L381S/Y, W382L/M, K383L/Q, K384A/G/S/T, Q385V, L387T/V, L388V, L389A/V, C390I/L/M/S/T, K392E/H/N/Q/R/S, V393A/G/I/L/M/S/T/Y, Y394K/N/S, W395E/F/I/L/M/Q/S/T, S396T, Q397E/F/G/H/I/K/L/M/R/S/T/V/W/Y, W398L/M, G402A/D/K/M/P/S/T, L403E/F/I/P, P404A/E/G/I/L/M/Q/R/S/T/V, S405A/G/L/P/Q/T/V/Y, G406A/D/E/H/M/N/P/R/T, K407A/D/G/L/N/P/Q/S/V/Y, A408D/G/L/N/S/V, D409E/G/K/N/P/Q/R/S/V/Y, E410V, G411E/H/K/R/T/V, D412M/N/P/Q/S/V, G413S/T, E414M/Q/R/V, A415G/P/S, S416A/G/H, C417A/G/S, F418C/S/T, L419A/I/M, I420C/L/V, Y424P, D425R, H426G, L427A/M, V428A/L/T, R429A/S, K430G/Q/S/W, S431G, K432I/V, H433A/P/T/V, N434G/L/P, M435F/L/N/T/V, P436C/G/H/L/S/T, T437A/H/P/S, G438P/V, S439C/L/P, N440D/M, M441E/T, G442E/L/M/R/V, E443A/D/P/V, S444G, P446D/G/H/S/T, K448R, I450V, K451R, R456M, C457T, P458K, R459L, G460A/D/H/L/Q/R/S/V, S461A/V, F462H/L/M/Y/, A463P/S, A465G, M466I/L, L467M, Q468H/I/M, M474H/I/L/V, R477A/E/G/I/L/Q/S/T/W, L479M/T/V, R480K/M/W, D481H, R484A/L/W/Y, E485I/L/V, K487R, M490A/I/L/V, D492A/C/G/H/M/R/S/Y, N493R, H494L/M/Q/R/T/V/Y, S495A/G/L/T, Y497L, M499L/N/R/S, T500A/N, R501K/L, G502M, I503L/R/V, Q504P/S/T, P506A/K/L, A507G, K508C/D/L/Q, I513V, E515G, L517I, G518S, L522A/F/M/V, E523A/D/H, F525G/H/I/R/T/V/W, or Y526E/G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence

corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

[0158] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 34, 60, 160, 162, 173, 174, 176, 177, 179, 187, 190, 191, 193, 194, 197, 203, 204, 205, 208, 222, 224, 230, 231, 236, 246, 251, 254, 260, 261, 262, 263, 264, 267, 273, 274, 277, 278, 295, 296, 297, 299, 300, 308, 310, 313, 318, 319, 325, 329, 334, 335, 339, 342, 343, 352, 353, 357, 361, 363, 367, 368, 376, 379, 381, 390, 396, 397, 402, 404, 405, 406, 407, 408, 409, 412, 416, 417, 420, 433, 437, 444, 451, 460, 462, 465, 466, 477, 480, 485, 487, 490, 492, 495, 503, 504, 508, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

[0159] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue 22P, 34Y, 60H, 160T, 162P, 173P, 174I/V/W, 176H, 177K, 179K, 187T/Y, 190R/T, 191F, 193S/T, 194L, 197M, 203M, 204M, 205L, 208E/G/Q, 222A, 224V, 230V, 231M/N, 236R, 246V, 251A, 254I/R, 260R, 261V, 262M, 263R/S, 264L, 267S, 273I/K/R/M, 274P, 277S, 278S, 295D, 296V, 297S, 299S, 300K/S, 308H, 310D, 313R, 318K/P/Q, 319T/V, 325V, 329L, 334S, 335V, 339S/T/V, 342T, 343L, 352T, 353L/R/V, 357I, 361V, 363P, 367Q, 368S, 376E, 379L, 381Y, 390M/S, 396T, 397K, 402S, 404S, 405Q, 406P, 407A/P, 408V, 409E, 412P, 416A, 417A, 420V, 433P, 437H, 444G, 451R, 460Q, 462L/M, 465G, 466I, 477A/L/Q, 480K, 485I, 487R, 490V, 492H/M, 495G, 503L, 504S, 508Q, or 525V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

[0160] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue F22P, D34Y, R60H, M160T, T162P, T173P, L174I/V/W, N176H, R177K, Q179K, I187T/Y, E/R190R/T, N191F, V193S/T, H194L, N197M, E203M, F204M, R205L, I208E/G/Q, K222A, T224V, I230V, S231M/N, G236R, E246V, Q251A, L/R254I/R, Q260R, S261V, F262M, H263R/S, A264L, R267S, V273I/K/R/M, G274P, D277S, K278S, T295D, L296V, K297S, T299S, T300K/S, Y308H, E310D, A313R, R318K/P/Q, A/T319T/V, W325V, Q329L, T334S, F335V, A339S/T/V, E342T, I343L, P352T, Q353L/R/V, V357I, I361V, S363P, E367Q, K368S, Q376E, M379L, L381Y, C390M/S, S396T, Q397K, G402S, P404S, S405Q, G406P, K/P407A/P, A408V, D409E, D412P, S416A, C417A, I420V, H433P, T437H, S444G, K451R, G460Q, F/M462L/M, A465G, M466I, R477A/L/Q, R480K, E485I, K487R, M490V, D/M492H/M, S495G, I503L, Q504S, K508Q, F525V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

[0161] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2, and one or more residue differences as compared to SEQ ID NO: 2 at amino acid position(s) 233/402, 296/384/412/415/451/477/480, 296/415/451/477/480, 296/451/477, 296/451/480, 384/412/451/480, 384/451/477, 402/404/460, 404, 404/431, 415/451/477/480/503, 451, 451/477, or 477. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2, and at least an amino acid residue difference(s), or amino acid residue 233P/402K, 296V/384S/412M/415P/451R/477Q/480W, 296V/415P/451R/477Q/480W, 296V/451R/477Q, 296V/451R/480W, 384A/412M/451R/480W, 384S/451R/477S, 402K/404A/460A, 402K/404A/460S, 404A, 404A/431G, 415P/451R/477Q/480W/503R, 451R, 451R/477Q, or 477Q. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2, and at least an amino acid residue difference(s), or amino acid residue E233P/G402K, L296V/K384S/D412M/A415P/K451R/R477Q/R480W, L296V/A415P/K451R/R477Q/R480W, L296V/K451R/R477Q, L296V/K451R/R480W, K384A/D412M/K451R/R480W, K384S/K451R/R477S, G402K/P404A/G460A, G402K/P404A/G460S, P404A, P404A/S431G, A415P/K451R/R477Q/R480W/I503R, K451R, K451R/R477Q, and R477Q.

[0162] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, or 526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414,

1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0163] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue 22P, 27P, 34D/Y, 41T, 42T, 43-/T, 45T, 52G, 60H, 92C, 105G, 133E, 152H, 160A/D/T/W, 161A/G/L/Q/T, 162P, 164Q, 169L, 173G/H/P, 174L/V/W, 175E/H/L/P/R/S/T/V, 176H/N, 177C/K, 179C/K/L/R/V, 182I/V, 183G/R/S/T/V, 184K/R, 186G/L/M/R/T/V, 187L/T/V/Y, 190A/F/G/H/L/M/N/Q/R/T/V, 191F/W/Y, 192A/H/S, 193A/G/I/Q/R/S/T/V/W, 194L/M, 195F/Q/R/T/W, 196D/H/K/L/Q/R/S/V, 197I/L/M/Q/R, 198E/H/P/Q/R/S/T, 199E/F/V, 200Q, 201D/E/P/Q/R/S, 202H/I/M/S/V, 203E/F/L/M/Q, 204M/V, 205L/R, 208A/D/E/G/M/N/Q/T, 212R, 213G, 218I, 219D/Q/S, 221I/Q/V, 222A/N, 223H/L/T, 224L/V, 227G/S, 229F/G/L/S, 230C/I/L/V, 231C/M/N/T, 232E/G, 233I/P, 234T, 235N/R, 236R/S, 237L, 238F/I/L/V, 239G/L/R/S, 241L, 242L, 244G/I/R/S, 246L/V, 249L/M/Q, 250G, 251A/E/G/M/R/S/W, 252E/H/L/M/R/S, 254I/N/Q/R, 259F, 260K/Q/R/S/T, 261G/R/S/T/V, 262L/M, 263A/D/H/K/R/S/T/W, 264A/L/R/S, 267G/S, 268L, 272R, 273E/G/I/K/L/M//N/Q/R/V, 274L/N/P/R/S/T/V, 275I/L/R/S, 277D/E/S/T, 278A/D/L/R/S, 280W, 281A/C/G/H/L/S, 282N, 288D, 290L, 291K/L, 292A, 293R, 294G/T, 295A/D/N/T, 296L/M/V, 297A/G/L/M/P/Q/S/T/V/W/Y, 298I, 299A/G/H/N/P/R/S, 300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, 301A/F/V, 302S, 303E/R/T, 304L, 307D/E/K/M/N/S, 308H/L/Y, 309H, 310D/S, 312M, 313A/H/Q/R/S/T, 314A/E/I/S/T, 315K/R, 316L, 317R, 318D/I/K/L/P/Q/R/S/T, , 319A/G/L/M/P/Q/T/V/W, 321R/T, 322A/E, 323T, 324A/T, 325G/S/V/Y, 328A/I, 329H/L/Q, 333N/S, 334S, 335V, 336C/T, 337S, 339A/I/S/T/V, 340L/M/T/V, 341L/T, 342S/T, 343I/L/M/R/T/V, 347A/S, 352A/E/L/M/N/S/T/V, 353E/K/L/R/T/V, 354G, 357I, 359A/C/F/V, 360V, 361I/L/V, 362A/M, 363G/P/T, 364S, 366D/F/H/P, 367E/N/Q/R/S/V, 368L/N/R/S, 370E/F/L/R, 371G/R, 376A/E/G/H/R/S/T/V, 377R, 378M, 379L/M/T, 380A/K/L/S/V, 381S/Y, 382L/M, 383L/Q, 384A/G/S/T, 385V, 387T/V, 388V, 389A/V, 390I/L/M/S/T, 392E/H/N/Q/R/S, 393A/G/I/L/M/S/T/Y, 394K/N/S, 395E/F/I/L/M/Q/S/T, S396T, 397E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 398L/M, 402A/D/K/M/P/S/T, 403E/F/I/P, 404A/E/G/I/L/M/Q/R/S/T/V, 405A/G/L/P/Q/S/T/V/Y, 406A/D/E/G/H/M/N/P/R/T, K407A/D/G/L/N/P/Q/S/V/Y, 408A/D/G/L/N/S/V, 409D/E/G/K/N/P/Q/R/S/V/Y, 410V, 411E/H/K/R/T/V, 412M/N/P/Q/S/V, 413S/T, 414M/Q/R/V, 415G/P/S, 416A/G/H/S, 417A/G/S, 418C/S/T, 419A/I/M, 420C/L/V, 424P, 425R, 426G, 427A/M, 428A/L/T, 429A/S, 430G/Q/S/W, 431G, 432I/V, 433A/P/T/V, 434G/L/P, 435F/L/N/T/V, 436C/G/H/L/S/T, 437A/H/P/S/T, 438P/V, 439C/L/P, 440D/M, 441E/T, 442E/L/M/R/V, 443A/D/P/V, 444G, 446D/G/H/S/T, 448R, 450V, 451R, 456M, 457T, 458K, 459L, 460A/D/H/L/Q/R/S/V, 461A/V, 462F/H/L/M/Y, 463P/S, 465G, 466I/L, 467M, 468H/I/M, 474H/I/L/V, 477A/E/G/I/L/Q/R/S/T/W, 479M/T/V, 480K/M/W, 481H, 484A/L/W/Y, 485E/I/L/V, 487K/R, 490A/I/L/V, 492A/C/G/H/M/R/S/Y, 493R, 494L/M/Q/R/T/V/Y, 495A/G/L/S/T, 497L, 499L/N/R/S, 500A/N, 501K/L, 502M, 503L/R/V, 504P/S/T, 506A/K/L, 507G, 508C/D/K/L/Q, 513V, 515G, 517I, 518S, 522A/F/M/V, 523A/D/H, 525G/H/I/R/T/V/W, or 526E/G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8,

70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0164] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue F22P, Q27P, D34D/Y, I41T, K42T, F43-/T, E45T, E52G, R60H, R92C, E105G, K133E, R152H, M160A/D/T/W, K161A/G/L/Q/T, T162P, S164Q, Q169L, T173G/H/P, L174L/V/W, N175E/H/L/P/R/S/T/V, N176H/N, R177C/K, Q179C/K/L/R/V, T182I/V, D183G/R/S/T/V, S184K/R, E186G/L/M/R/T/V, I187L/T/V/Y, E190A/F/G/H/L/M/N/Q/R/T/V, N191F/W/Y, Y192A/H/S, V193A/G/I/Q/R/S/T/V/W, F194L/M, K195F/Q/R/T/W, G196D/H/K/L/Q/R/S/V, N197I/L/M/Q/R, D198E/H/P/Q/R/S/T, D199E/F/V, K200Q, A201D/E/P/Q/R/S, L202H/I/M/S/V, E203E/F/L/M/Q, F204M/V, R205L/R, I208A/D/E/G/M/N/Q/T, K212R, S213G, V218I, R219D/Q/S, L221I/Q/V, K222A/N, D223H/L/T, T224L/V, L227G/S, W229F/G/L/S, I230C/I/L/V, S231C/M/N/T, D232E/G, E233I/P, V234T, K235N/R, G236R/S, I237L, M238F/I/L/V, E239G/L/R/S, I241L, I242L, V244G/I/R/S, E246L/V, E249L/M/Q, V250G, Q251A/E/G/M/R/S/W, A252E/H/L/M/R/S, L254I/N/Q/R, Y259F, Q260K/Q/R/S/T, S261G/R/S/T/V, F262L/M, H263A/D/H/K/R/S/T/W, A264A/L/R/S, R267G/S, V268L, G272R, V273E/G/I/K/L/M//N/Q/R/V, G274L/N/P/R/S/T/V, T275I/L/R/S, D277D/E/S/T, K278A/D/L/R/S, Y280W, Q281A/C/G/H/L/S, M282N, E288D, V290L, R291K/L, S292A, S293R, K294G/T, T295A/D/N/T, L296L/M/V, K297A/G/L/M/P/Q/S/T/V/W/Y, L298I, T299A/G/H/N/P/R/S, T300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, W301A/F/V, A302S, K303E/R/T, V304L, T307D/E/K/M/N/S, Y308H/L/Y, Y309H, E310D/S, L312M, A313A/H/Q/R/S/T, R314A/E/I/S/T, G315K/R, V316L, T317R, R318D/I/K/L/P/Q/R/S/T, , A319A/G/L/M/P/Q/T/V/W, A321R/T, D322A/E, A323T, I324A/T, W325G/S/V/Y, V328A/I, Q329H/L/Q, A333N/S, T334S, F335V, D336C/T, P337S, A339A/I/S/T/V, I340L/M/T/V, V341L/T, E342S/T, I343I/L/M/R/T/V, Q347A/S, P352A/E/L/M/N/S/T/V, Q353E/K/L/R/T/V, S354G, V357I, L359A/C/F/V, L360V, I361I/L/V, S362A/M, S363G/P/T, P364S, A366D/F/H/P, E367E/N/Q/R/S/V, K368L/N/R/S, Q370E/F/L/R, E371G/R, Q376A/E/G/H/R/S/T/V, K377R, V378M, M379L/M/T, R380A/K/L/S/V, L381S/Y, W382L/M, K383L/Q, K384A/G/S/T, Q385V, L387T/V, L388V, L389A/V, C390I/L/M/S/T, K392E/H/N/Q/R/S, V393A/G/I/L/M/S/T/Y, Y394K/N/S, W395E/F/I/L/M/Q/S/T, S396T, Q397E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, W398L/M, G402A/D/K/M/P/S/T, L403E/F/I/P, P404A/E/G/I/L/M/Q/R/S/T/V, S405A/G/L/P/Q/S/T/V/Y, G406A/D/E/G/H/M/N/P/R/T, K407A/D/G/L/N/P/Q/S/V/Y, A408A/D/G/L/N/S/V, D409D/E/G/K/N/P/Q/R/S/V/Y, E410V, G411E/H/K/R/T/V, D412M/N/P/Q/S/V, G413S/T, E414M/Q/R/V, A415G/P/S, S416A/G/H/S, C417A/G/S, F418C/S/T, L419A/I/M, I420C/L/V, Y424P, D425R, H426G, L427A/M, V428A/L/T, R429A/S, K430G/Q/S/W, S431G, K432I/V, H433A/P/T/V, N434G/L/P, M435F/L/N/T/V, P436C/G/H/L/S/T, T437A/H/P/S/T, G438P/V, S439C/L/P, N440D/M, M441E/T, G442E/L/M/R/V, E443A/D/P/V, S444G, P446D/G/H/S/T, K448R, I450V, K451R, R456M, C457T, P458K, R459L, G460A/D/H/L/Q/R/S/V, S461A/V, F462F/H/L/M/Y/, A463P/S, A465G, M466I/L, L467M, Q468H/I/M, M474H/I/L/V, R477A/E/G/I/L/Q/R/S/T/W, L479M/T/V, R480K/M/W, D481H, R484A/L/W/Y, E485E/I/L/V, K487K/R, M490A/I/L/V, D492A/C/G/H/M/R/S/Y, N493R, H494L/M/Q/R/T/V/Y, S495A/G/L/S/T, Y497L,

M499L/N/R/S, T500A/N, R501K/L, G502M, I503L/R/V, Q504P/S/T, P506A/K/L, A507G, K508C/D/K/L/Q, I513V, E515G, L517I, G518S, L522A/F/M/V, E523A/D/H, F525G/H/I/R/T/V/W, or Y526E/G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0165] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 34, 60, 160, 162, 173, 174, 176, 177, 179, 187, 190, 191, 193, 194, 197, 203, 204, 205, 208, 222, 224, 230, 231, 236, 246, 251, 254, 260, 261, 262, 263, 264, 267, 273, 274, 277, 278, 295, 296, 297, 299, 300, 308, 310, 313, 318, 319, 325, 329, 334, 335, 339, 342, 343, 352, 353, 357, 361, 363, 367, 368, 376, 379, 381, 390, 396, 397, 402, 404, 405, 406, 407, 408, 409, 412, 416, 417, 420, 433, 437, 444, 451, 460, 462, 465, 466, 477, 480, 485, 487, 490, 492, 495, 503, 504, 508, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0166] In some embodiments, the engineered terminal nucleotidyl transferase comprises an amino acid sequence comprising at least an amino acid residue difference or amino acid residue 22P, 34Y, 60H, 160T, 162P, 173P, 174I/V/W, 176H, 177K, 179K, 187T/Y, 190R/T, 191F, 193S/T, 194L, 197M, 203M, 204M, 205L/R, 208E/G/Q, 222A, 224V, 230I/V, 231M/N, 236R, 246V, 251A, 254I/R, 260R, 261V, 262M, 263R/S, 264L, 267S, 273I/K/R/M, 274P, 277D/S, 278S, 295D, 296L/V, 297S, 299S, 300K/S, 308H, 310D, 313R, 318K/P/Q, 319T/V, 325V, 329L, 334S, 335V, 339S/T/V, 342T, 343L, 352T, 353L/R/V, 357I, 361V, 363P, 367Q, 368S, 376E, 379L, 381Y, 390M/S, 396T, 397K, 402S, 404S, 405Q, 406P, 407A/P, 408A/V, 409D/E, 412P, 416A, 417A, 420V, 433P, 437H, 444G, 451R, 460Q, 462L/M, 465G, 466I, 477A/L/Q, 480K, 485I, 487R, 490V, 492H/M, 495G, 503L, 504S, 508Q, or 525V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0167] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ ID NO: 8 at amino acid positions(s) 92/273/277/462, 267/313/314, 267/313/314/408/485, 267/313/367/485, 267/313/406, 267/314/408/441, 267/367/485, 267/406/408/485, 273/277, 273/277/381, 273/277/381/404/462/492, 273/277/381/429, 273/277/381/462, 273/277/381/462/492, 273/277/381/492, 273/277/429, 273/277/429/492, 273/277/462, 273/381, 273/381/492, 273/462, 273/462/492, 277/381, 277/381/429/462/492, 277/381/429/492, 277/381/462/492, 277/381/492, 277/429/462/492, 277/429/492, 277/492, 309/313/314/412/485, 381/462/492, and 381/492, or combinations thereof. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ ID NO: 8, or amino acid residues selected from 92C/273R/277T/462M, 267S/313S/314A/408V/485L, 267S/313S/367S/485L, 267S/313S/406H, 267S/313T/314A, 267S/314A/408V/441E, 267S/367S/485L, 267S/406H/408V/485L, 273R/277S, 273R/277S/381Y, 273R/277S/381Y/404S/462M/492M, 273R/277S/381Y/429A, 273R/277S/381Y/462M, 273R/277S/381Y/462M/492M, 273R/277S/381Y/462M/492Y, 273R/277S/429A, 273R/277S/429A/492S, 273R/277S/462M, 273R/277T, 273R/277T/381Y, 273R/277T/381Y/429A, 273R/277T/381Y/492M, 273R/277T/429A, 273R/277T/462M, 273R/381Y, 273R/381Y/492M, 273R/462M, 273R/462M/492S, 273R/462M/492Y, 277S/381Y, 277S/381Y/429A/462M/492M, 277S/381Y/429A/492M, 277S/381Y/462M/492M, 277S/381Y/462M/492S, 277S/429A/462M/492S, 277S/429A/492Y, 277S/492S, 277T/381Y, 277T/381Y/429A/462M/492M, 277T/381Y/492M, 277T/381Y/492S, 309H/313S/314A/412N/485L, 381Y/462M/492Y, and 381Y/492S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ ID NO: 8, or amino acid residues selected from R92C/V273R/D277T/F462M, R267S/A313S/R314A/A408V/E485L, R267S/A313S/E367S/E485L, R267S/A313S/G406H, R267S/A313T/R314A, R267S/R314A/A408V/M441E, R267S/E367S/E485L, R267S/G406H/A408V/E485L, V273R/D277S, V273R/D277S/L381Y, V273R/D277S/L381Y/P404S/F462M/D492M, V273R/D277S/L381Y/R429A, V273R/D277S/L381Y/F462M, V273R/D277S/L381Y/F462M/D492M, V273R/D277S/L381Y/F462M/D492Y, V273R/D277S/R429A, V273R/D277S/R429A/D492S, V273R/D277S/F462M, V273R/D277T, V273R/D277T/L381Y, V273R/D277T/L381Y/R429A, V273R/D277T/L381Y/D492M, V273R/D277T/R429A, V273R/D277T/F462M, V273R/L381Y, V273R/L381Y/D492M, V273R/F462M, V273R/F462M/D492S, V273R/F462M/D492Y, D277S/L381Y, D277S/L381Y/R429A/F462M/D492M,

D277S/L381Y/R429A/D492M, D277S/L381Y/F462M/D492M, D277S/L381Y/F462M/D492S, D277S/R429A/F462M/D492S, D277S/R429A/D492Y, D277S/D492S, D277T/L381Y, D277T/L381Y/R429A/F462M/D492M, D277T/L381Y/D492M, D277T/L381Y/D492S, Y309H/A313S/R314A/D412N/E485L, L381Y/F462M/D492Y, and L381Y/D492S.

[0168] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ lD NO: 8 at amino acid positions(s) 186, 193, 196, 198, 198/515, 274, 394, and 409, or combinations thereof. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ ID NO: 8, or amino acid residues selected from 186R, 193Q, 193T, 193W, 196K, 198H, 198Q, 198R, 198R/515G, 198S, 274R, 394S, and 409R, or combinations thereof. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 8, and one or more residue differences as compared to SEQ ID NO: 8, or amino acid residues selected from E186R, V193Q, V193T, V193W, G196K, D198H, D198Q, D198R, D198R/E515G, D198S, G274R, Y394S, and D409R, or combinations thereof.

[0169] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 70, or to a reference sequence corresponding to SEQ ID NO: 70, and one or more residue differences as compared to SEQ ID NO: 70 at amino acid positions(s) 190, 190/208/267/274, 190/208/267/274/309/417, 190/208/267/274/417/462, 190/208/274/309/313/417/462, 190/208/309/462, 190/267/274, 190/274/417, 190/274/417/477, 208/274/309/313/430, 267/274/477, 273, 274, 274/309, 274/309/313/417/462, 274/309/417/477, 274/313, and 274/462. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 8, or to a reference sequence corresponding to SEQ ID NO: 70, and one or more residue differences as compared to SEQ ID NO: 8, or amino acid residues selected from 190A, 190A/208E/267S/274P, 190A/208E/309H/462H, 190A/208G/267S/274P/417A/462H, 190A/274P/417A, 190R/208G/267S/274P/309H/417A, 190R/208G/274P/309H/313S/417A/462H, 190R/267S/274P, 190R/274P/417A/477R, 208E/274P/309H/313S/430Q, 267S/274P/477R, 273V, 274P, 274P/309H,

274P/309H/313S/417A/462H, 274P/309H/417A/477R, 274P/313S, and 274P/462H. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 70, or to a reference sequence corresponding to SEQ ID NO: 70, and one or more residue differences as compared to SEQ ID NO: 70, or amino acid residues selected from E190A, E190A/I208E/R267S/G274P, E190A/I208E/Y309H/M462H, E190A/I208G/R267S/G274P/C417A/M462H, E190A/G274P/C417A, E190R/I208G/R267S/G274P/Y309H/C417A, E190R/I208G/G274P/Y309H/A313S/C417A/M462H, E190R/R267S/G274P, E190R/G274P/C417A/Q477R, I208E/G274P/Y309H/A313S/K430Q, R267S/G274P/Q477R, R273V, G274P, G274P/Y309H, G274P/Y309H/A313S/C417A/M462H, G274P/Y309H/C417A/Q477R, G274P/A313S, and G274P/M462H.

[0170] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148 at amino acid positions(s) 183/208/309/313/407/462, 208/343/407, 208/407, and 309. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from 183R/208E/309H/313S/407P/462H, 208D/343L/407P, 208E/343L/407P, 208E/407P, or 309H. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from D183R/I208E/Y309H/A313S/K407P/M462H, I208D/I343L/K407P, I208E/I343L/K407P, I208E/K407P, and Y309H.

[0171] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148 at amino acid positions(s) 190, 268, 275, 303, 397, 409, 415, 417, and 477. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a

reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from 190H, 268L, 275L, 275S, 303T, 397V, 409P, 415G, 415P, 417S, and 477R. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from R190H, V268L, T275L, T275S, K303T, Q397V, D409P, A415G, A415P, C417S, and Q477R.

[0172] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148 at amino acid positions 186, 304, 318, 347, 353, 409, 416, 417, and 420. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from 186L, 186T, 186V, 304L, 318L, 347A, 353L, 409P, 416H, 417S, and 420L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 148, or to a reference sequence corresponding to SEQ ID NO: 148, and one or more residue differences as compared to SEQ ID NO: 148, or amino acid residues selected from E186L, E186T, E186V, V304L, R318L, Q347A, Q353L, D409P, S416H, C417S, and I420L.

[0173] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 184, or to a reference sequence corresponding to SEQ ID NO: 184, and one or more residue differences as compared to SEQ ID NO: 184 at amino acid positions 193/274/411/465/525, 193/408/465, 193/465, 274/352/353/465, 281/411/465, 343/408/465, 343/411, 411, 411/525, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 184, or to a reference sequence corresponding to SEQ ID NO: 184, and one or more residue differences as compared to SEQ ID NO: 184, or amino acid residues selected from 193S/274N/411T/465G/525H, 193S/408L/465G, 193S/465G,

274N/352M/353T/465G, 281S/411T/465G, 343I/408L/465G, 343I/411T, 411T, 411T/525H, and 525H. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 184, or to a reference sequence corresponding to SEQ ID NO: 184, and one or more residue differences as compared to SEQ ID NO: 184, or amino acid residues selected from V193S/P274N/G411T/A465G/F525H, V193S/A408L/A465G, V193S/A465G, P274N/P352M/Q353T/A465G, Q281S/G411T/A465G, L343I/A408L/A465G, L343I/G411T, G411T, G411T/F525H, and F525H.

[0174] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234 at amino acid positions 275, 299, 300, 392, 393, and 397. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from 275I, 299G, 299P, 299R, 299S, 300F, 300G, 300I, 300K, 300L, 300M, 300N, 300Q, 300R, 300Y, 392H, 392N, 393G, 393Y, 397F, 397H, 397I, 397K, 397M, 397R, 397V, 397W, and 397Y. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from T275I, T299G, T299P, T299R, T299S, T300F, T300G, T300I, T300K, T300L, T300M, T300N, T300Q, T300R, T300Y, K392H, K392N, V393G, V393Y, Q397F, Q397H, Q397I, Q397K, Q397M, Q397R, Q397V, Q397W, and Q397Y.

[0175] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234 at amino acid positions 299, 300, 392, 393, 395, and 397. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or

amino acid residues selected from 299A, 299G, 299N, 299R, 299S, 300A, 300E, 300F, 300L, 300M, 300N, 300S, 300V, 300Y, 392E, 392N, 392Q, 392S, 393A, 393I, 393L, 393M, 393S, 393T, 395F, 395I, 395L, 395M, 395Q, 395T, 397E, 397L, and 397M. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from T299A, T299G, T299N, T299R, T299S, T300A, T300E, T300F, T300L, T300M, T300N, T300S, T300V, T300Y, K392E, K392N, K392Q, K392S, V393A, V393I, V393L, V393M, V393S, V393T, W395F, W395I, W395L, W395M, W395Q, W395T, Q397E, Q397L, and Q397M.

[0176] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234 at amino acid positions 307, 359, 405, 420, 492, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from 307S, 359F, 405T, 420V, 492H, and 525T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from T307S, L359F, S405T, I420V, M492H, and F525T.

[0177] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234 at amino acid positions 298, 308, 312, 318, 319, 322, 328, 359, 361, 379, 382, 389, 390, 392, 394, 398, 404, 405, 409, 410, 411, 412, 414, 415, 417, 419, 420, 457, 458, 466, 468, 492, 493, 494, 495, 513, 517, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more

residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from 298I, 308L, 312M, 318I, 319M, 319T, 322A, 328I, 359V, 361L, 379T, 382M, 389A, 390I, 392R, 394K, 398M, 404I, 404L, 404R, 405Y, 409G, 409R, 409S, 409Y, 410V, 411H, 411R, 411V, 412V, 414R, 415S, 417G, 419I, 419M, 420C, 457T, 458K, 466L, 468H, 468I, 468M, 492C, 492G, 492H, 492R, 492S, 493R, 494Q, 495G, 495L, 495T, 513V, 517I, and 525I. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 234, or to a reference sequence corresponding to SEQ ID NO: 234, and one or more residue differences as compared to SEQ ID NO: 234, or amino acid residues selected from L298I, Y308L, L312M, R318I, A319M, A319T, D322A, V328I, L359V, I361L, M379T, W382M, L389A, C390I, K392R, Y394K, W398M, S404I, S404L, S404R, S405Y, D409G, D409R, D409S, D409Y, E410V, G411H, G411R, G411V, D412V, E414R, A415S, C417G, L419I, L419M, I420C, C457T, P458K, M466L, Q468H, Q468I, Q468M, M492C, M492G, M492H, M492R, M492S, N493R, H494Q, S495G, S495L, S495T, I513V, L517I, and F525I.

[0178] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256 at amino acid positions 307, 319/410/495, 319/466/492/495, 319/492/495, 325/359/404/409, 359/404/405, 405, 405/409, 410, 412, 415, 419, 442/457, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from 307S, 319T/410V/495G, 319T/466L/492H/495G, 319T/492H/495G, 325G/359F/404T/409R, 359F/404R/405T, 405T, 405T/409R, 410V, 412V, 415P, 419M, 442R/457T, and 525T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from T307S, A319T/E410V/S495G, A319T/M466L/M492H/S495G, A319T/M492H/S495G, W325G/L359F/S404T/D409R, L359F/S404R/S405T, S405T, S405T/D409R, E410V, D412V, A415P, L419M, G442R/C457T, and F525T.

[0179] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID

NO: 256, and one or more residue differences as compared to SEQ ID NO: 256 at amino acid positions 323, 336, 339, 362, 363, 363/434, 368, 380, 383, 384, 402, 403, 426, 428, 430, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 459, 460, 497, 499, 501, 504, and 506. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 660%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from 323T, 336C, 336T, 339T, 362M, 363P, 363P/434P, 368S, 380A, 380L, 380S, 380V, 383L, 384G, 384T, 402D, 402S, 402T, 403I, 426G, 428A, 430S, 432I, 432V, 433T, 434G, 434L, 434P, 435F, 435L, 435T, 436C, 436H, 436L, 437A, 437P, 438P, 439C, 439L, 439P, 440M, 441T, 442L, 443A, 443P, 443V, 459L, 460R, 460S, 460V, 497L, 499R, 501L, 504P, 506A, and 506L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from A323T, D336C, D336T, A339T, S362M, S363P, S363P/N434P, K368S, R380A, R380L, R380S, R380V, K383L, K384G, K384T, G402D, G402S, G402T, L403I, H426G, V428A, K430S, K432I, K432V, H433T, N434G, N434L, N434P, M435F, M435L, M435T, P436C, P436H, P436L, T437A, T437P, G438P, S439C, S439L, S439P, N440M, M441T, G442L, E443A, E443P, E443V, R459L, G460R, G460S, G460V, Y497L, M499R, R501L, Q504P, P506A, and P506L.

[0180] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256 at amino acid positions 329, 336, 339, 362, 363, 363/434, 368, 380, 381, 383, 384, 385, 397, 402, 425, 427, 428, 430, 432, 433, 435, 436, 437, 438, 439, 443, 459, 460, 497, 499, 500, 501, 506, 508, and 518. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from 329H, 336C, 339I, 339T, 362A, 363P/434P, 363T, 368L, 368S, 380A, 380L, 380V, 381S, 383Q, 384T, 385V, 397T, 402A, 402D, 402M, 402P, 402S, 402T, 425R, 427A, 428T, 430W, 432I, 433V, 435T, 436C, 436G, 437A, 438P, 439P, 443V, 459L, 460L, 460S, 460V, 497L, 499L, 500A, 501L, 506A, 506L, 508L, and 518S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12

to carboxy terminal of SEQ ID NO: 256, or to a reference sequence corresponding to SEQ ID NO: 256, and one or more residue differences as compared to SEQ ID NO: 256, or amino acid residues selected from Q329H, D336C, A339I, A339T, S362A, S363P/N434P, S363T, K368L, K368S, R380A, R380L, R380V, Y381S, K383Q, K384T, Q385V, K397T, G402A, G402D, G402M, G402P, G402S, G402T, D425R, L427A, V428T, K430W, K432I, H433V, M435T, P436C, P436G, T437A, G438P, S439P, E443V, R459L, G460L, G460S, G460V, Y497L, M499L, T500A, R501L, P506A, P506L, K508L, and G518S.

[0181] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 478, or to a reference sequence corresponding to SEQ ID NO: 478, and one or more residue differences as compared to SEQ ID NO: 478 at amino acid positions 299/302/363/402/405/416, 299/363/402, 299/363/405/416, 299/363/459, 299/402/416/501, 299/459, 302/363/402/405, 302/402/459, 402/416/459, and 459. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 478, or to a reference sequence corresponding to SEQ ID NO: 478, and one or more residue differences as compared to SEQ ID NO: 478, or amino acid residues selected from 299S/302S/363P/402S/405T/416A, 299S/363P/402S, 299S/363P/405T/416A, 299S/363P/459L, 299S/402S/416A/501L, 299S/459L, 302S/363P/402S/405T, 302S/402S/459L, 402S/416A/459L, and 459L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 478, or to a reference sequence corresponding to SEQ ID NO: 478, and one or more residue differences as compared to SEQ ID NO: 478, or amino acid residues selected from T299S/A302S/S363P/G402S/S405T/S416A, T299S/S363P/G402S, T299S/S363P/S405T/S416A, T299S/S363P/R459L, T299S/G402S/S416A/R501L, T299S/R459L, A302S/S363P/G402S/S405T, A302S/G402S/R459L, G402S/S416A/R459L, and R459L.

[0182] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668 at amino acid positions 302/416/497, 368, 368/416/435/436/501, and 416. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from 302S/416A/497L, 368S,

368S/416A/435L/436H/501L, and 416A. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from A302S/S416A/Y497L, K368S, K368S/S416A/M435L/P436H/R501L, and S416A.

[0183] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668 at amino acid positions 339/368/436 and 416. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from 339T/368S/436L, and 416A. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from A339T/K368S/P436L, and S416A.

[0184] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668 at amino acid positions 184, 191, 222, 224, 232, 236, 246, 251, 288, 291, 319, 444, 485, and 490. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from 184R, 191F, 191W, 191Y, 222A, 224L, 232E, 236S, 246V, 251R, 288D, 291K, 319G, 319W, 444G, 485I, and 490I. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12

to carboxy terminal of SEQ ID NO: 668, or to a reference sequence corresponding to SEQ ID NO: 668, and one or more residue differences as compared to SEQ ID NO: 668, or amino acid residues selected from S184R, N191F, N191W, N191Y, K222A, T224L, D232E, G236S, E246V, Q251R, E288D, R291K, T319G, T319W, S444G, E485I, and M490I.

[0185] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 682, or to a reference sequence corresponding to SEQ ID NO: 682, and one or more residue differences as compared to SEQ ID NO: 682 at amino acid positions 191, 368, 485, and 490. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 682, or to a reference sequence corresponding to SEQ ID NO: 682, and one or more residue differences as compared to SEQ ID NO: 682, or amino acid residues selected from 191F, 368S, 485I, and 490I. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 682, or to a reference sequence corresponding to SEQ ID NO: 682, and one or more residue differences as compared to SEQ ID NO: 682, or amino acid residues selected from N191F, K368S, E485I, and M490I.

[0186] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730 at amino acid positions 191/222/236/246/291/368/444, 191/222/244/246/291/368/490, 191/222/244/246/368/490, 191/222/244/246/368/495, 191/222/244/291/368/444, 191/222/246/368/444, 191/222/291/368, 191/222/291/368/490, 191/222/368, 191/222/368/444/490, 191/222/368/490, 191/236/244/291/368/490, 191/236/291/368, 191/236/291/368/490, 191/246/291/368/490, 191/246/368/490, 191/291/368/444, 191/291/368/444/490, 191/368/444, 191/368/444/490, 191/368/490, 222/236/246/291/368/444/490, 222/244/291/368/444, 222/246/368/444, 222/246/490, 222/291/368, 222/291/368/444, 222/291/368/444/490, 222/368/444/490, 222/368/490, 236/246/291/368/444/490, 236/246/368, 244/246/291/368, 244/246/368/444, 244/246/368/490, 244/291/368/490, 246/291/296/368/444/490, 246/291/368, 246/291/368/490, 246/368/444, 368, 368/444, and 368/490. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or

amino acid residues selected from 191F/222A/236S/246V/291K/368S/444G, 191F/222A/244R/246V/291K/368S/490I, 191F/222A/244R/246V/368S/490I, 191F/222A/244R/246V/368S/495S, 191F/222A/244R/291K/368S/444G, 191F/222A/246V/368S/444G, 191F/222A/291K/368S, 191F/222A/291K/368S/490V, 191F/222A/368S, 191F/222A/368S/444G/490V, 191F/222A/368S/490I, 191F/236S/244R/291K/368S/490V, 191F/236S/291K/368S, 191F/236S/291K/368S/490V, 191F/246V/291K/368S/490I, 191F/246V/368S/490V, 191F/291K/368S/444G, 191F/291K/368S/444G/490I, 191F/368S/444G, 191F/368S/444G/490V, 191F/368S/490I, 191F/368S/490V, 222A/236S/246V/291K/368S/444G/490V, 222A/244R/291K/368S/444G, 222A/246V/368S/444G, 222A/246V/490I, 222A/291K/368S, 222A/291K/368S/444G, 222A/291K/368S/444G/490V, 222A/368S/444G/490I, 222A/368S/444G/490V, 222A/368S/490V, 236S/246V/291K/368S/444G/490V, 236S/246V/368S, 244R/246V/291K/368S, 244R/246V/368S/444G, 244R/246V/368S/490V, 244R/291K/368S/490V, 246V/291K/296M/368S/444G/490V, 246V/291K/368S, 246V/291K/368S/490V, 246V/368S/444G, 368S, 368S/444G, and 368S/490V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or amino acid residues selected from N191F/K222A/G236S/E246V/R291K/K368S/S444G, N191F/K222A/V244R/E246V/R291K/K368S/M490I, N191F/K222A/V244R/E246V/K368S/M490I, N191F/K222A/V244R/E246V/K368S/G495S, N191F/K222A/V244R/R291K/K368S/S444G, N191F/K222A/E246V/K368S/S444G, N191F/K222A/R291K/K368S, N191F/K222A/R291K/K368S/M490V, N191F/K222A/K368S, N191F/K222A/K368S/S444G/M490V, N191F/K222A/K368S/M490I, N191F/G236S/V244R/R291K/K368S/M490V, N191F/G236S/R291K/K368S, N191F/G236S/R291K/K368S/M490V, N191F/E246V/R291K/K368S/M490I, N191F/E246V/K368S/M490V, N191F/R291K/K368S/S444G, N191F/R291K/K368S/S444G/M490I, N191F/K368S/S444G, N191F/K368S/S444G/M490V, N191F/K368S/M490I, N191F/K368S/M490V, K222A/G236S/E246V/R291K/K368S/S444G/M490V, K222A/V244R/R291K/K368S/S444G, K222A/E246V/K368S/S444G, K222A/E246V/M490I, K222A/R291K/K368S, K222A/R291K/K368S/S444G, K222A/R291K/K368S/S444G/M490V, K222A/K368S/S444G/M490I, K222A/K368S/S444G/M490V, K222A/K368S/M490V, G236S/E246V/R291K/K368S/S444G/M490V, G236S/E246V/K368S, V244R/E246V/R291K/K368S, V244R/E246V/K368S/S444G, V244R/E246V/K368S/M490V, V244R/R291K/K368S/M490V, E246V/R291K/V296M/K368S/S444G/M490V, E246V/R291K/K368S, E246V/R291K/K368S/M490V, E246V/K368S/S444G, K368S, K368S/S444G, and K368S/M490V.

[0187] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730 at amino acid positions 161,

173, 197, 201, 208, 262, 343, 379, 382, 416, 429, 494, 508, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or amino acid residues selected from 161G, 161T, 173G, 197M, 201E, 208A, 262M, 343V, 379L, 382L, 416G, 429S, 494R, 508D, and 525W. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or amino acid residues selected from K161G, K161T, T173G, N197M, A201E, E208A, F262M, L343V, M379L, W382L, A416G, R429S, H494R, K508D, and F525W.

[0188] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730 at amino acid positions 161, 164, 201, 213, 223, 263, 333, 337, 411, 429, 463, 467, 479/485, 484/485, 502, 507, 522, 523, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or amino acid residues selected from 161G, 161Q, 164Q, 201E, 213G, 223T, 263A, 333N, 333S, 337S, 411E, 429S, 463P, 467M, 479V/485V, 484W/485E, 502M, 507G, 522M, 522V, 523D, and 525R. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 730, or to a reference sequence corresponding to SEQ ID NO: 730, and one or more residue differences as compared to SEQ ID NO: 730, or amino acid residues selected from K161G, K161Q, S164Q, A201E, S213G, D223T, H263A, A333N, A333S, P337S, G411E, R429S, A463P, L467M, L479V/I485V, R484W/I485E, G502M, A507G, L522M, L522V, E523D, and F525R.

[0189] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 742, or to a reference sequence corresponding to SEQ ID NO: 742, and one or more residue differences as compared to SEQ ID NO: 742 at amino acid positions 161,

161/197/208/262/416, 161/208, 161/208/262/411/416/429, 161/208/262/411/416/467, 161/208/467, 161/262, 161/262/411, 161/262/411/416, 161/262/411/416/467, 161/262/411/467, 161/262/416, 161/262/416/467, 161/262/429, 161/262/467, 161/416/442, 161/429, 197, 197/208, 197/208/262, 197/208/262/411/429/467, 197/208/411, 197/208/411/416/467, 197/208/416, 197/208/467, 197/262, 197/262/467, 208, 208/262, 208/262/411/416, 208/262/416/467, 208/262/429, 208/411/416, 208/411/467, 208/416, 208/416/429, 262, 262/411, 262/411/416, 262/411/416/429, 262/416, 262/416/467, 411/416, and 416. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 742, or to a reference sequence corresponding to SEQ ID NO: 742, and one or more residue differences as compared to SEQ ID NO: 742, or amino acid residues selected from 161G, 161G/197M/208G/262M/416G, 161G/208G, 161G/208G/262M/411E/416G/429S, 161G/208G/262M/411E/416G/467M, 161G/208G/467M, 161G/262L/429S, 161G/262M, 161G/262M/411E, 161G/262M/411E/416G, 161G/262M/411E/416G/467M, 161G/262M/411E/467M, 161G/262M/416G, 161G/262M/416G/467M, 161G/262M/467M, 161G/416G/442R, 161G/429S, 197M, 197M/208G, 197M/208G/262M, 197M/208G/262M/411E/429S/467M, 197M/208G/411E, 197M/208G/411E/416G/467M, 197M/208G/416G, 197M/208G/467M, 197M/262M, 197M/262M/467M, 208G, 208G/262M, 208G/262M/411E/416G, 208G/262M/416G/467M, 208G/262M/429S, 208G/411E/416G, 208G/411E/467M, 208G/416G, 208G/416G/429S, 262M, 262M/411E, 262M/411E/416G, 262M/411E/416G/429S, 262M/416G, 262M/416G/467M, 411E/416G, and 416G. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 742, or to a reference sequence corresponding to SEQ ID NO: 742, and one or more residue differences as compared to SEQ ID NO: 742, or amino acid residues selected from K161G, K161G/N197M/E208G/F262M/A416G, K161G/E208G, K161G/E208G/F262M/G411E/A416G/R429S, K161G/E208G/F262M/G411E/A416G/L467M, K161G/E208G/L467M, K161G/F262L/R429S, K161G/F262M, K161G/F262M/G411E, K161G/F262M/G411E/A416G, K161G/F262M/G411E/A416G/L467M, K161G/F262M/G411E/L467M, K161G/F262M/A416G, K161G/F262M/A416G/L467M, K161G/F262M/L467M, K161G/A416G/G442R, K161G/R429S, N197M, N197M/E208G, N197M/E208G/F262M, N197M/E208G/F262M/G411E/R429S/L467M, N197M/E208G/G411E, N197M/E208G/G411E/A416G/L467M, N197M/E208G/A416G, N197M/E208G/L467M, N197M/F262M, N197M/F262M/L467M, E208G, E208G/F262M, E208G/F262M/G411E/A416G, E208G/F262M/A416G/L467M, E208G/F262M/R429S, E208G/G411E/A416G, E208G/G411E/L467M, E208G/A416G, E208G/A416G/R429S, F262M, F262M/G411E, F262M/G411E/A416G, F262M/G411E/A416G/R429S, F262M/A416G, F262M/A416G/L467M, G411E/A416G, and A416G.

[0190] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932 at amino acid positions 262/317, 262/329, 262/339, 262/403, 262/462, 262/492, 262/526, and 387. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932, or amino acid residues selected from 262M/317R, 262M/329L, 262M/339V, 262M/403E, 262M/403F, 262M/403P, 262M/462L, 262M/492A, 262M/526E, and 387V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932, or amino acid residues selected from F262M/T317R, F262M/Q329L, F262M/A339V, F262M/L403E, F262M/L403F, F262M/L403P, F262M/M462L, F262M/H492A, F262M/Y526E, and L387V.

[0191] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 734, or to a reference sequence corresponding to SEQ ID NO: 734, and one or more residue differences as compared to SEQ ID NO: 734 at amino acid positions 183, 186, 193, 264, 294, 297, 313, 314, 315, 347, 352, 353, 359, 389, 398, 405, 406, and 409. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 734, or to a reference sequence corresponding to SEQ ID NO: 734, and one or more residue differences as compared to SEQ ID NO: 734, or amino acid residues selected from 183R, 183S, 186G, 186L, 186M, 186T, 186V, 193R, 264L, 294G, 297A, 297S, 313H, 313S, 314A, 314S, 315K, 315R, 347S, 352A, 352N, 353K, 359A, 359C, 389V, 398L, 405P, 406R, and 409P. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 734, or to a reference sequence corresponding to SEQ ID NO: 734, and one or more residue differences as compared to SEQ ID NO: 734, or amino acid residues selected from D183R, D183S, E186G, E186L, E186M, E186T, E186V, S193R, A264L, K294G, K297A, K297S, A313H, A313S, R314A, R314S, G315K, G315R, Q347S, P352A, P352N, Q353K, L359A, L359C, L389V, W398L, S405P, G406R, and D409P.

[0192] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932 at amino acid positions 184/262, 262/340, 262/363, 262/371, 262/371/443, 262/377, and 262/387. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932, or amino acid residues selected from 184K/262M, 262M/340V, 262M/363G, 262M/371G/443D, 262M/371R, 262M/377R, and 262M/387T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 932, or to a reference sequence corresponding to SEQ ID NO: 932, and one or more residue differences as compared to SEQ ID NO: 932, or amino acid residues selected from S184K/F262M, F262M/I340V, F262M/P363G, F262M/E371G/E443D, F262M/E371R, F262M/K377R, and F262M/L387T.

[0193] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 992, or to a reference sequence corresponding to SEQ ID NO: 992, and one or more residue differences as compared to SEQ ID NO: 992 at amino acid positions 60/193/264/297, 60/263/264/297/300/525, 193/297, 208/273/294/407, 208/273/295, 273, and 297. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 992, or to a reference sequence corresponding to SEQ ID NO: 992, and one or more residue differences as compared to SEQ ID NO: 992, or amino acid residues selected from 60H/193T/264L/297S, 60H/263A/264L/297S/300S/525W, 193T/297A, 208E/273L/294T/407G, 208E/273M/295N, 273L, and 297S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 992, or to a reference sequence corresponding to SEQ ID NO: 992, and one or more residue differences as compared to SEQ ID NO: 992, or amino acid residues selected from R60H/S193T/A264L/K297S, R60H/H263A/A264L/K297S/T300S/F525W, S193T/K297A, G208E/R273L/K294T/P407G, G208E/R273M/T295N, R273L, and K297S.

[0194] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144 at amino acid positions 229, 230, 231, 233, 234, 237, 238, 241, 250, 251, 252, 260/264, and 264. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144, or amino acid residues selected from 229F, 230C, 230L, 231C, 231M, 231T, 233I, 234T, 237L, 238F, 238L, 238V, 241L, 250G, 251E, 251G, 251M, 251R, 251S, 251W, 252M, 252S, 260R/264A, and 264A. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144, or amino acid residues selected from W229F, I230C, I230L, S231C, S231M, S231T, E233I, V234T, I237L, M238F, M238L, M238V, I241L, V250G, Q251E, Q251G, Q251M, Q251R, Q251S, Q251W, A252M, A252S, Q260R/L264A, and L264A.

[0195] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144 at amino acid positions 329, 329/339/340, 329/339/340/371/387, 329/339/340/403, 329/339/340/403/526, 329/339/371/387, 329/339/403/443, 329/526, 339, 339/340/387/526, 339/340/428, 339/403, 339/526, 371/403/526, and 443/526. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144, or amino acid residues selected from 329L, 329L/339V/340V, 329L/339V/340V/371G/387T, 329L/339V/340V/403E, 329L/339V/340V/403E/526E, 329L/339V/371G/387T, 329L/339V/403E/443D, 329L/526E, 339V, 339V/340V/387V/526E, 339V/340V/428L, 339V/403E, 339V/526E, 371G/403E/526E, and 443D/526E. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1144, or to a reference sequence corresponding to SEQ ID

NO: 1144, and one or more residue differences as compared to SEQ ID NO: 1144, or amino acid residues selected from Q329L, Q329L/A339V/I340V, Q329L/A339V/I340V/E371G/L387T, Q329L/A339V/I340V/L403E, Q329L/A339V/I340V/L403E/Y526E, Q329L/A339V/E371G/L387T, Q329L/A339V/L403E/E443D, Q329L/Y526E, A339V, A339V/I340V/L387V/Y526E, A339V/I340V/V428L, A339V/L403E, A339V/Y526E, E371G/L403E/Y526E, and E443D/Y526E.

[0196] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1212, or to a reference sequence corresponding to SEQ ID NO: 1212, and one or more residue differences as compared to SEQ ID NO: 1212 at amino acid positions 184/329/403/404/492, 184/403/404/526, 329, 329/526, 340, 340/460, 376, and 492. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1212, or to a reference sequence corresponding to SEQ ID NO: 1212, and one or more residue differences as compared to SEQ ID NO: 1212, or amino acid residues selected from 184K/329L/403E/404G/492A, 184K/403F/404G/526E, 329L, 329L/526E, 340M, 340V/460V, 376S, and 492A. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1212, or to a reference sequence corresponding to SEQ ID NO: 1212, and one or more residue differences as compared to SEQ ID NO: 1212, or amino acid residues selected from S184K/Q329L/L403E/S404G/H492A, S184K/L403F/S404G/Y526E, Q329L, Q329L/Y526E, I340M, I340V/G460V, Q376S, and H492A.

[0197] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236 at amino acid positions 174, 190, 198, 201, 202, 212, 263, 297, 318, 319, 322/329, 325/329, 329, 343, 379, 412, 415, 419, 448, 450, 456, 463, 477, 479, 480, 484, 523, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236, or amino acid residues selected from 174W, 190A, 190F, 190L, 190T, 190V, 198E, 201D, 202M, 202S, 212R, 263A, 263S, 263W, 297G, 297P, 318Q, 319Q, 322E/329Q, 325G/329Q, 325S/329Q, 325Y/329Q, 329Q, 343R, 343T, 343V, 379L, 412Q, 415G, 419A, 448R, 450V, 456M, 463S, 477T, 479V, 480M, 484A, 523D, 525I, 525R, and 525V. In some embodiments, the engineered TnT polypeptide comprises

an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236, or amino acid residues selected from L174W, R190A, R190F, R190L, R190T, R190V, D198E, A201D, L202M, L202S, K212R, H263A, H263S, H263W, S297G, S297P, R318Q, T319Q, D322E/L329Q, W325G/L329Q, W325S/L329Q, W325Y/L329Q, L329Q, L343R, L343T, L343V, M379L, D412Q, A415G, L419A, K448R, I450V, R456M, A463S, Q477T, L479V, R480M, R484A, E523D, F525I, F525R, and F525V.

[0198] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236 at amino acid positions 175, 179, 183, 192, 194, 196, 199, 203, 208, 264, 280, 281, 295, 300, 313, 314, 352, 353, 354, 389, 404, 406, 407, 408, and 409. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236, or amino acid residues selected from 175V, 179L, 183V, 192A, 194L, 196H, 199F, 203M, 208Q, 264R, 280W, 281C, 281G, 281H, 281L, 281S, 295A, 300L, 300N, 313H, 313R, 314T, 352E, 352L, 353L, 353V, 354G, 389V, 404E, 404L, 404Q, 404V, 406N, 406T, 407L, 407V, 408L, and 409E. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1236, or to a reference sequence corresponding to SEQ ID NO: 1236, and one or more residue differences as compared to SEQ ID NO: 1236, or amino acid residues selected from N175V, Q179L, D183V, Y192A, F194L, G196H, D199F, E203M, G208Q, L264R, Y280W, Q281C, Q281G, Q281H, Q281L, Q281S, T295A, T300L, T300N, A313H, A313R, R314T, P352E, P352L, Q353L, Q353V, S354G, L389V, S404E, S404L, S404Q, S404V, G406N, G406T, P407L, P407V, A408L, and D409E.

[0199] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264 at amino acid positions 190/263/295/300/342/393, 190/300/525, 263/295/300/420, 263/295/420, 264/300/420, 264/379, 264/420, 264/420/525, 295/379/420, 295/420, 300/379/420, 300/420, 313, 313/379/525, 342, 342/420, 379, 379/420, 420, and 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence

having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from 190G/263S/295A/300S/342S/393T, 190G/300S/525V, 263S/295A/300S/420V, 263S/295A/420V, 264S/300S/420V, 264S/379L, 264S/420V, 264S/420V/525V, 295A/379L/420V, 295A/420V, 300S/379L/420V, 300S/420V, 313R, 313R/379L/525V, 342S, 342S/420V, 379L, 379L/420V, 420V, and 525V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from T190G/H263S/T295A/T300S/E342S/V393T, T190G/T300S/F525V, H263S/T295A/T300S/I420V, H263S/T295A/I420V, L264S/T300S/I420V, L264S/M379L, L264S/I420V, L264S/I420V/F525V, T295A/M379L/I420V, T295A/I420V, T300S/M379L/I420V, T300S/I420V, A313R, A313R/M379L/F525V, E342S, E342S/I420V, M379L, M379L/I420V, I420V, and F525V.

[0200] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264 at amino acid positions 174, 190, 190/193, 190/194, 260, 353, 392, 393, 395, 405, 407, 408, 411, and 418. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from 174V, 190M, 190R/193I, 190R/194M, 260R, 353L, 353V, 392Q, 393T, 395E, 395M, 395T, 405Q, 407Q, 408N, 411K, and 418C. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from L174V, T190M, T190R/T193I, T190R/F194M, Q260R, Q353L, Q353V, K392Q, V393T, W395E, W395M, W395T, S405Q, P407Q, A408N, G411K, and F418C.

[0201] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264 at amino acid positions 190, 190/193, 190/194, 353, 405, 407, 408, and 409. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from 190R, 190R/193G, 190R/193S, 190R/193V, 190R/194M, 353L, 405Q, 405V, 407G, 407Y, 408V, and 409V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1264, or to a reference sequence corresponding to SEQ ID NO: 1264, and one or more residue differences as compared to SEQ ID NO: 1264, or amino acid residues selected from T190R, T190R/T193G, T190R/T193S, T190R/T193V, T190R/F194M, Q353L, S405Q, S405V, P407G, P407Y, A408V, and D409V.

[0202] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1414, or to a reference sequence corresponding to SEQ ID NO: 1414, and one or more residue differences as compared to SEQ ID NO: 1414 at amino acid positions 169/409, 194, 203/208/295/308/409, 203/208/295/389/409, 263/300/353, 295/308/409, 300, and 352/353. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1414, or to a reference sequence corresponding to SEQ ID NO: 1414, and one or more residue differences as compared to SEQ ID NO: 1414, or amino acid residues selected from 169L/409E, 194L, 203M/208Q/295A/308H/409E, 203M/208Q/295D/308H/409E, 203M/208Q/295D/389V/409E, 263S/300N/353V, 295A/308H/409E, 300N, and 352A/353V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1414, or to a reference sequence corresponding to SEQ ID NO: 1414, and one or more residue differences as compared to SEQ ID NO: 1414, or amino acid residues selected from Q169L/D409E, F194L, E203M/G208Q/T295A/Y308H/D409E, E203M/G208Q/T295D/Y308H/D409E, E203M/G208Q/T295D/L389V/D409E, H263S/T300N/Q353V, T295A/Y308H/D409E, T300N, and P352A/Q353V.

[0203] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 1504, or to a reference sequence corresponding to SEQ ID NO: 1504, and one or more residue differences as compared to SEQ ID NO: 1504 at amino acid positions 174/260/263/353/405/408, 174/353, 174/405/407/420, 174/407/409/420, 260/353, 260/353/420, 263/352/353/405/408/420, 263/353/420, 263/420, 353/420, 405/408/409/420, 407/408/420, 407/409/420, and 420. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 174V/260R/263S/353L/405Q/408V, 174V/353V, 174V/405Q/407Y/420V, 174V/407Y/409D/420V, 260R/353V, 260R/353V/420V, 263S/352A/353V/405Q/408V/420V, 263S/353V/420V, 263S/420V, 353L/420V, 353V/420V, 405Q/408V/409D/420V, 407S/409D/420V, 407Y/408V/420V, and 420V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1504, or to a reference sequence corresponding to SEQ ID NO: 1504, and one or more residue differences as compared to SEQ ID NO: 1504, or amino acid residues selected from L174V/Q260R/H263S/Q353L/S405Q/A408V, L174V/Q353V, L174V/S405Q/P407Y/I420V, L174V/P407Y/E409D/I420V, Q260R/Q353V, Q260R/Q353V/I420V, H263S/P352A/Q353V/S405Q/A408V/I420V, H263S/Q353V/I420V, H263S/I420V, Q353L/I420V, Q353V/I420V, S405Q/A408V/E409D/I420V, P407S/E409D/I420V, P407Y/A408V/I420V, and I420V.

[0204] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1504, or to a reference sequence corresponding to SEQ ID NO: 1504, and one or more residue differences as compared to SEQ ID NO: 1504 at amino acid positions 133/197/203, 192, 197/203, 261, 263, 281, 281/440, 295/297, 295/301/308, 308, 308/314, 322, 328, 389, 390, 394, 404/409, 405/409, 461, and 484. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1504, or to a reference sequence corresponding to SEQ ID NO: 1504, and one or more residue differences as compared to SEQ ID NO: 1504, or amino acid residues selected from 133E/197Q/203E, 192H, 197R/203E, 261G, 261R, 261V, 263K, 263R, 281G, 281S/440D, 295T/297G, 295T/297P, 295T/301V/308Y, 308L, 308Y, 308Y/314I, 322E, 328A, 389V, 390L, 390M, 394N, 394S, 404I/409D, 404M/409D, 405A/409D, 405V/409D, 461A, and 484W. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1504, or to a reference sequence corresponding to SEQ ID NO: 1504, and one or more residue differences as compared to SEQ ID NO: 1504, or amino acid residues selected from K133E/N197Q/M203E, Y192H, N197R/M203E, S261G, S261R, S261V, H263K, H263R, Q281G, Q281S/N440D, D295T/S297G, D295T/S297P, D295T/W301V/H308Y, H308L, H308Y,

H308Y/R314I, D322E, V328A, L389V, C390L, C390M, Y394N, Y394S, S404I/E409D, S404M/E409D, S405A/E409D, S405V/E409D, S461A, and R484W.

[0205] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522 at amino acid positions 52, 175, 182, 193, 195, 196, 201, and 278. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from 52G, 175E, 175S, 175T, 182V, 193A, 193G, 193V, 195R, 195T, 196V, 201S, and 278R. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from E52G, N175E, N175S, N175T, T182V, T193A, T193G, T193V, K195R, K195T, G196V, A201S, and K278R.

[0206] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522 at amino acid positions 173, 193, 195, 196, 201, 272, 275, 278, 282, 297, 299, 301, 314, 412, 418, 461, 462, 479, and 494. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from 173P, 193R, 195F, 195W, 196R, 201P, 201R, 272R, 275R, 275S, 278R, 282N, 297A, 297P, 297T, 297V, 297W, 299G, 299H, 299P, 301A, 314A, 412S, 412V, 418S, 418T, 461V, 462F, 479M, 479T, 494L, 494M, 494Q, and 494V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from T173P, T193R, K195F, K195W, G196R, A201P, A201R, G272R,

T275R, T275S, K278R, M282N, S297A, S297P, S297T, S297V, S297W, S299G, S299H, S299P, W301A, R314A, D412S, D412V, F418S, F418T, S461V, L462F, L479M, L479T, H494L, H494M, H494Q, and H494V.

[0207] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522 at amino acid positions 174/194/195/197/319/361/420, 174/194/197/260/263/319/353/409/420, 174/194/197/260/263/353/409/420, 194/195/197/319, 194/195/319/420, 194/195/361, 194/197, 194/197/319/361/420, 194/319, 194/319/361/420, 194/319/420, 195/319/361/420, 195/319/420, 197/420, 300/325/406, 300/353/420/525, 300/406/409, 319/420, 325/406, and 361/420. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from 174L/194L/195Q/197M/319V/361V/420V, 174L/194L/197M/260Q/263H/319V/353V/409D/420V, 174L/194L/197M/260Q/263H/353V/409D/420V, 194L/195Q/197M/319V, 194L/195Q/319V/420V, 194L/195Q/361V, 194L/197M, 194L/197M/319V/361V/420V, 194L/319V, 194L/319V/361V/420V, 194L/319V/420V, 195Q/319V/361V/420V, 195Q/319V/420V, 197M/420V, 300N/325V/406D, 300N/353V/420V/525T, 300N/406D/409D, 319V/420V, 325V/406D, and 361V/420V. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1522, or to a reference sequence corresponding to SEQ ID NO: 1522, and one or more residue differences as compared to SEQ ID NO: 1522, or amino acid residues selected from V174L/F194L/K195Q/N197M/T319V/I361V/I420V, V174L/F194L/N197M/R260Q/S263H/T319V/L353V/E409D/I420V, V174L/F194L/N197M/R260Q/S263H/L353V/E409D/I420V, F194L/K195Q/N197M/T319V, F194L/K195Q/T319V/I420V, F194L/K195Q/I361V, F194L/N197M, F194L/N197M/T319V/I361V/I420V, F194L/T319V, F194L/T319V/I361V/I420V, F194L/T319V/I420V, K195Q/T319V/I361V/I420V, K195Q/T319V/I420V, N197M/I420V, T300N/W325V/G406D, T300N/L353V/I420V/V525T, T300N/G406D/E409D, T319V/I420V, W325V/G406D, and I361V/I420V.

[0208] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1702, or to a reference sequence corresponding to SEQ ID NO: 1702, and one or more residue differences as compared to SEQ ID NO: 1702 at amino acid positions

261/409, 297, 300, and 409. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1702, or to a reference sequence corresponding to SEQ ID NO: 1702, and one or more residue differences as compared to SEQ ID NO: 1702, or amino acid residues selected from 261V/409D, 297P, 300N, and 409D. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1702, or to a reference sequence corresponding to SEQ ID NO: 1702, and one or more residue differences as compared to SEQ ID NO: 1702, or amino acid residues selected from S261V/E409D, S297P, T300N, and E409D.

[0209] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742 at amino acid positions 52/201/390, 175/193/297/300, 175/297/300, 175/297/300/462, 175/300, 175/300/314, 175/300/442, 193/195/297/300/314, 193/297/300/314/442, 195/297/300/462, 221/297/300, and 297/300. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from 52G/201S/390M, 175S/193G/297V/300N, 175S/297V/300N, 175S/297V/300N/462F, 175S/300N, 175S/300N/314A, 175S/300N/442R, 193G/195T/297V/300N/314A, 193G/297V/300N/314A/442R, 195T/297A/300N/462F, 195T/297V/300N/462F, 221Q/297A/300N, 297A/300N, and 297V/300N. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from E52G/A201S/C390M, N175S/T193G/S297V/T300N, N175S/S297V/T300N, N175S/S297V/T300N/L462F, N175S/T300N, N175S/T300N/R314A, N175S/T300N/G442R, T193G/K195T/S297V/T300N/R314A, T193G/S297V/T300N/R314A/G442R, K195T/S297A/T300N/L462F, K195T/S297V/T300N/L462F, L221Q/S297A/T300N, S297A/T300N, and S297V/T300N.

[0210] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742 at amino acid positions 152/281, 182, 196, 197, 261, 281, 297, 303, 307, 319, 397, 404/409, 405/409, 406/409, 409, 463, 474, and 477. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from 152H/281S, 182V, 196D, 196L, 196S, 197I, 261T, 281S, 297T, 303R, 307N, 319P, 397S, 404E/409E, 404G/409E, 405S/409E, 406A/409E, 406P/409E, 409E, 463P, 474L, and 477S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from R152H/Q281S, T182V, G196D, G196L, G196S, M197I, V261T, Q281S, S297T, K303R, T307N, V319P, K397S, S404E/D409E, S404G/D409E, Q405S/D409E, G406A/D409E, G406P/D409E, D409E, A463P, M474L, and Q477S.

[0211] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742 at amino acid positions 152/281, 190, 197, 208, 274, 281, 297, 299, 303, 307, 308, 313, 319, 321, 360, 404/409, 406/409, 417, 460, 461, 477, 479, 492, and 495. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from 152H/281S, 190R, 197I, 208E, 208T, 274S, 281A, 281G, 281H, 281S, 297T, 299N, 303R, 303T, 307D, 307E, 307M, 308Y, 313A, 319G, 319L, 319M, 319P, 321T, 360V, 404E/409E, 404M/409E, 404V/409E, 406A/409E, 406M/409E, 406P/409E, 417G, 460D, 461A, 477E, 479M, 479T, 492G, and 495A. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1742, or to a reference sequence corresponding to SEQ ID NO: 1742, and one or more residue differences as compared to SEQ ID NO: 1742, or amino acid residues selected from R152H/Q281S, T190R, M197I, Q208E, Q208T, P274S, Q281A, Q281G, Q281H, Q281S, S297T, S299N, K303R, K303T, T307D, T307E, T307M, H308Y, R313A, V319G, V319L,

V319M, V319P, A321T, L360V, S404E/D409E, S404M/D409E, S404V/D409E, G406A/D409E, G406M/D409E, G406P/D409E, C417G, G460D, S461A, Q477E, L479M, L479T, H492G, and G495A.

[0212] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1928, or to a reference sequence corresponding to SEQ ID NO: 1928, and one or more residue differences as compared to SEQ ID NO: 1928 at amino acid positions 161/251/408, 183/522, 196/231/522, 231/416/474, 273, 273/274/325/416/522, 416, or 416/474. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1928, or to a reference sequence corresponding to SEQ ID NO: 1928, and one or more residue differences as compared to SEQ ID NO: 1928, or amino acid residues selected from 161G/251M/408A, 183T/522V, 196Q/231M/522V, 231M/416S/474L, 273M, 273M/274R/325V/416G/522V, 416S, or 416S/474L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1928, or to a reference sequence corresponding to SEQ ID NO: 1928, and one or more residue differences as compared to SEQ ID NO: 1928, or amino acid residues selected from K161G/Q251M/V408A, D183T/L522V, G196Q/S231M/L522V, S231M/A416S/M474L, R273M, R273M/P274R/W325V/A416G/L522V, A416S, or A416S/M474L.

[0213] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944 at amino acid positions 231/408 and 406/463. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944, or amino acid residues selected from 231M/408A and 406P/463P. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944, or amino acid residues selected from S231M/V408A and G406P/A463P.

[0214] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944 at amino acid positions 231, 273, 367, 407, 424, 427, 429, 430, or 499. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944, or amino acid residues selected from 231N, 273I, 273V, 367N, 367Q, 407D, 424P, 427M, 429S, 430G, 499N, and 499R. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1944, or to a reference sequence corresponding to SEQ ID NO: 1944, and one or more residue differences as compared to SEQ ID NO: 1944, or amino acid residues selected from S231N, M273I, M273V, E367N, E367Q, P407D, Y424P, L427M, R429S, K430G, M499N, and M499R.

[0215] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1946, or to a reference sequence corresponding to SEQ ID NO: 1946, and one or more residue differences as compared to SEQ ID NO: 1946 at amino acid positions 196, 196/273, 196/273/325, 196/273/325/335, 196/273/325/335/367, 196/273/430, 196/335, 196/335/367, 196/335/367/430, 196/367/479, 196/430, 273, 273/274, 273/274/367/430, 273/325/335/367, 273/325/430, 273/335/430, 273/367, 273/430, 273/430/479, 274, 274/325/335/479, 274/430, 325/335, 325/335/417, 325/335/417/430/479, 325/367, 335, 335/367, 335/367/479, 335/430, 335/479, 367, 367/430, 367/479, 417, 417/430/479, 430, and 479. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1946, or to a reference sequence corresponding to SEQ ID NO: 1946, and one or more residue differences as compared to SEQ ID NO: 1946, or amino acid residues selected from 196Q, 196Q/273I/325V, 196Q/273I/325V/335V, 196Q/273I/430G, 196Q/335V, 196Q/335V/367R/430G, 196Q/367R/479M, 196Q/430G, 196S/273I, 196S/273I/325V/335V/367Q, 196S/335V/367R, 196S/367R/479M, 273I, 273I/274R, 273I/274R/367R/430G, 273I/325V/335V/367Q, 273I/325V/430G, 273I/335V/430G, 273I/367R, 273I/430G, 273I/430G/479M, 274R, 274R/325V/335V/479M, 274R/430G, 325V/335V, 325V/335V/417S, 325V/335V/417S/430G/479M, 325V/367Q, 325V/367R, 335V, 335V/367Q, 335V/367R/479M, 335V/430G, 335V/479M, 367Q, 367Q/479M, 367R, 367R/430G, 417S, 417S/430G/479M, 430G, and 479M. In some embodiments, the

engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1946, or to a reference sequence corresponding to SEQ ID NO: 1946, and one or more residue differences as compared to SEQ ID NO: 1946, or amino acid residues selected from G196Q, G196Q/M273I/W325V, G196Q/M273I/W325V/F335V, G196Q/M273I/K430G, G196Q/F335V, G196Q/F335V/E367R/K430G, G196Q/E367R/L479M, G196Q/K430G, G196S/M273I, G196S/M273I/W325V/F335V/E367Q, G196S/F335V/E367R, G196S/E367R/L479M, M273I, M273I/P274R, M273I/P274R/E367R/K430G, M273I/W325V/F335V/E367Q, M273I/W325V/K430G, M273I/F335V/K430G, M273I/E367R, M273I/K430G, M273I/K430G/L479M, P274R, P274R/W325V/F335V/L479M, P274R/K430G, W325V/F335V, W325V/F335V/C417S, W325V/F335V/C417S/K430G/L479M, W325V/E367Q, W325V/E367R, F335V, F335V/E367Q, F335V/E367R/L479M, F335V/K430G, F335V/L479M, E367Q, E367Q/L479M, E367R, E367R/K430G, C417S, C417S/K430G/L479M, K430G, and L479M.

[0216] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984at amino acid positions 231, 231/273, 231/273/380/417/500, 231/273/506, 231/380/417/501, 231/500/501/506, or 231/501. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984, or amino acid residues selected from 231N, 231N/273K, 231N/273K/380K/417S/500N, 231N/273K/506K, 231N/273M, 231N/380K/417S/501K, 231N/500N/501K/506K, and 231N/501K. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984, or amino acid residues selected from M231N, M231N/I273K, M231N/I273K/R380K/C417S/T500N, M231N/I273K/P506K, M231N/I273M, M231N/R380K/C417S/R501K, M231N/T500N/R501K/P506K, and M231N/R501K.

[0217] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984at amino acid positions

187, 190, 259, 260, 263, 273, 296, 300, 314, 343, 361, 367, 376, 384, 389, 390, 393, 395, 416, 417, 428, or 446. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984, or amino acid residues selected from 187L, 187T, 187V, 187Y, 190Q, 190V, 259F, 260K, 260S, 260T, 263R, 273M, 296L, 300A, 300K, 314E, 343M, 343V, 361I, 367E, 376E, 384S, 389V, 390S, 390T, 393T, 395F, 395S, 416G, 417A, 428L, 446D, 446G, 446H, 446S, and 446T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 1984, or to a reference sequence corresponding to SEQ ID NO: 1984, and one or more residue differences as compared to SEQ ID NO: 1984, or amino acid residues selected from I187L, I187T, I187V, I187Y, T190Q, T190V, Y259F, R260K, R260S, R260T, S263R, I273M, V296L, N300A, N300K, A314E, L343M, L343V, V361I, Q367E, Q376E, K384S, L389V, M390S, M390T, V393T, W395F, W395S, A416G, C417A, V428L, P446D, P446G, P446H, P446S, and P446T.

[0218] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2064, or to a reference sequence corresponding to SEQ ID NO: 2064, and one or more residue differences as compared to SEQ ID NO: 2064 at amino acid positions 161, 162, 174, 201, 219, 223, 244, 246, 252, 254, 318, 433, 437, 503, or 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2064, or to a reference sequence corresponding to SEQ ID NO: 2064, and one or more residue differences as compared to SEQ ID NO: 2064, or amino acid residues selected from 161L, 162P, 174W, 201Q, 219D, 223H, 244R, 246L, 252L, 254N, 254Q, 254R, 318K, 433A, 437H, 503V, and 525T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2064, or to a reference sequence corresponding to SEQ ID NO: 2064, and one or more residue differences as compared to SEQ ID NO: 2064, or amino acid residues selected from K161L, T162P, V174W, A201Q, R219D, D223H, V244R, E246L, A252L, L254N, L254Q, L254R, R318K, H433A, T437H, I503V, and V525T.

[0219] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 2104, or to a reference sequence corresponding to SEQ ID NO: 2104, and one or more residue differences as compared to SEQ ID NO: 2104 at amino acid positions 174/199/389/390, 174/231, 174/231/260/273/389/390, 174/231/263/273/343, 174/231/263/389, 174/231/389/390/446, 174/231/390, 174/259/260/263/273/361/389/390/446, 174/259/273/343/361/389, 174/263/389/390, 174/273/343/389/390/446, 187/190/231/252/273/296/300/353/417, 187/190/252/273/300/417, 187/190/273/353, 187/231/252/273/296/300/353, 187/231/273/296/300/353, 187/231/296/300/353, 187/231/300/353, 187/273/353, 190/231/273, 196/199/231/263/273/389, 199/231/260/263/361/389/446, 231, 231/259/263/273, 231/273, 259/263/273/389/390, 260/273/446, 263/273/343/446, 273, 273/353, 273/389/446, or 273/390. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2104, or to a reference sequence corresponding to SEQ ID NO: 2104, and one or more residue differences as compared to SEQ ID NO: 2104, or amino acid residues selected from 174L/199V/389V/390T, 174L/231N, 174L/231N/260K/273K/389V/390T, 174L/231N/263R/389V, 174L/231N/263T/273K/343V, 174L/231N/263T/389V, 174L/231N/389V/390T/446S, 174L/231N/390T, 174L/259F/260K/263R/273K/361I/389V/390T/446S, 174L/259F/273K/343V/361I/389V, 174L/263R/389V/390T, 174L/273K/343V/389V/390T/446S, 187V/190Q/252M/273K/300K/417S, 187V/190Q/273K/353V, 187V/231N/252E/273K/296L/300K/353R, 187V/231N/300K/353V, 187V/273K/353V, 187Y/190Q/231N/252M/273K/296L/300K/353V/417S, 187Y/231N/273K/296L/300K/353R, 187Y/231N/296L/300K/353R, 190Q/231N/273K, 196Q/199V/231N/263T/273K/389V, 199V/231N/260K/263T/361I/389V/446S, 231N, 231N/259F/263T/273K, 231N/273K, 259F/263R/273K/389V/390T, 260K/273K/446S, 263T/273K/343V/446S, 273K, 273K/353V, 273K/389V/446S, and 273K/390T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2104, or to a reference sequence corresponding to SEQ ID NO: 2104, and one or more residue differences as compared to SEQ ID NO: 2104, or amino acid residues selected from V174L/D199V/L389V/S390T, V174L/M231N, V174L/M231N/R260K/I273K/L389V/S390T, V174L/M231N/S263R/L389V, V174L/M231N/S263T/I273K/L343V, V174L/M231N/S263T/L389V, V174L/M231N/L389V/S390T/P446S, V174L/M231N/S390T, V174L/Y259F/R260K/S263R/I273K/V361I/L389V/S390T/P446S, V174L/Y259F/I273K/L343V/V361I/L389V, V174L/S263R/L389V/S390T, V174L/I273K/L343V/L389V/S390T/P446S, I187V/T190Q/A252M/I273K/N300K/C417S, I187V/T190Q/I273K/L353V, I187V/M231N/A252E/I273K/V296L/N300K/L353R, I187V/M231N/N300K/L353V, I187V/I273K/L353V, I187Y/T190Q/M231N/A252M/I273K/V296L/N300K/L353V/C417S, I187Y/M231N/I273K/V296L/N300K/L353R, I187Y/M231N/V296L/N300K/L353R, T190Q/M231N/I273K,

G196Q/D199V/M231N/S263T/I273K/L389V, D199V/M231N/R260K/S263T/V361I/L389V/P446S, M231N, M231N/Y259F/S263T/I273K, M231N/I273K, Y259F/S263R/I273K/L389V/S390T, R260K/I273K/P446S, S263T/I273K/L343V/P446S, I273K, I273K/L353V, I273K/L389V/P446S, and I273K/S390T.

[0220] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210 at amino acid positions 161/162/254/417, 161/162/318/446, 174/244/273/390/437/503, 174/244/437/525, 174/273/503, 201/376, 433, or 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210, or amino acid residues selected from 161L/162P/254R/417A, 161L/162P/318K/446S, 174W/244G/437H/525T, 174W/244R/273K/390T/437H/503L, 174W/273K/503L, 201Q/376E, 433P, and 525T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210, or amino acid residues selected from K161L/T162P/L254R/C417A, K161L/T162P/R318K/P446S, V174W/V244G/T437H/V525T, V174W/V244R/I273K/S390T/T437H/I503L, V174W/I273K/I503L, A201Q/Q376E, H433P, and V525T.

[0221] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210 at amino acid positions 303, 397, or 466. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210, or amino acid residues selected from 303E, 397S, and 466I. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue

differences as compared to SEQ ID NO: 2210, or amino acid residues selected from K303E, K397S, and M466I.

[0222] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210 at amino acid positions 278, 290, 297, 303, 397, or 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210, or amino acid residues selected from 278S, 290L, 297L, 297Y, 303E, 397G, 397S, and 525G. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2210, or to a reference sequence corresponding to SEQ ID NO: 2210, and one or more residue differences as compared to SEQ ID NO: 2210, or amino acid residues selected from K278S, V290L, S297L, S297Y, K303E, K397G, K397S, and V525G.

[0223] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2256, or to a reference sequence corresponding to SEQ ID NO: 2256, and one or more residue differences as compared to SEQ ID NO: 2256 at amino acid positions 22, 22/162/201/244/254/318/376/417/433/437/525, 22/201/376, 22/244, 22/244/254, 22/244/433/525, 22/318/376/433/525, 22/318/376/446, 22/376/417/437/446/525, 161/162/201/244/318/376/417/437, 161/162/201/244/376, 162, 162/201/318/376/417/433/525, 162/201/318/376/433/437/446, 162/244/254/433, 162/244/318/376/417/433/437, 162/254, 162/254/318/376/417/433, 162/254/376/417/433, 162/376/525, 162/433, 201/244/318/376, 201/244/376/433/525, 201/318/376/446, 244/318/376/417, 244/318/376/417/433/437/525, 318/376, 318/376/433, 433, or 437. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2256, or to a reference sequence corresponding to SEQ ID NO: 2256, and one or more residue differences as compared to SEQ ID NO: 2256, or amino acid residues selected from 22P, 22P/162P/201Q/244G/254R/318K/376E/417A/433P/437H/525T, 22P/201Q/376E, 22P/244G, 22P/244G/254R, 22P/244G/433P/525T, 22P/318K/376E/433P/525T, 22P/318K/376E/446S, 22P/376E/417A/437H/446S/525T, 161L/162P/201Q/244G/318K/376E/417A/437H, 161L/162P/201Q/244G/376E, 162P, 162P/201Q/318K/376E/417A/433P/525T,

162P/201Q/318K/376E/433P/437H/446S, 162P/244G/254R/433P, 162P/244G/318K/376E/417A/433P/437H, 162P/254R, 162P/254R/318K/376E/417A/433P, 162P/254R/376E/417A/433P, 162P/376E/525T, 162P/433P, 201Q/244G/318K/376E, 201Q/244G/376E/433P/525T, 201Q/318K/376E/446S, 244G/318K/376E/417A, 244G/318K/376E/417A/433P/437H/525T, 318K/376E, 318K/376E/433P, 433P, and 437H. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2256, or to a reference sequence corresponding to SEQ ID NO: 2256, and one or more residue differences as compared to SEQ ID NO: 2256, or amino acid residues selected from F22P, F22P/T162P/A201Q/V244G/L254R/R318K/Q376E/C417A/H433P/T437H/V525T, F22P/A201Q/Q376E, F22P/V244G, F22P/V244G/L254R, F22P/V244G/H433P/V525T, F22P/R318K/Q376E/H433P/V525T, F22P/R318K/Q376E/P446S, F22P/Q376E/C417A/T437H/P446S/V525T, K161L/T162P/A201Q/V244G/R318K/Q376E/C417A/T437H, K161L/T162P/A201Q/V244G/Q376E, T162P, T162P/A201Q/R318K/Q376E/C417A/H433P/V525T, T162P/A201Q/R318K/Q376E/H433P/T437H/P446S, T162P/V244G/L254R/H433P, T162P/V244G/R318K/Q376E/C417A/H433P/T437H, T162P/L254R, T162P/L254R/R318K/Q376E/C417A/H433P, T162P/L254R/Q376E/C417A/H433P, T162P/Q376E/V525T, T162P/H433P, A201Q/V244G/R318K/Q376E, A201Q/V244G/Q376E/H433P/V525T, A201Q/R318K/Q376E/P446S, V244G/R318K/Q376E/C417A, V244G/R318K/Q376E/C417A/H433P/T437H/V525T, R318K/Q376E, R318K/Q376E/H433P, H433P, and T437H.

[0224] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280 at amino acid positions 22/27/437/466, 22/34/278/318/437/466, 22/466, 41/42/43/397, 43/244/397, 45/397, or 397. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280, or amino acid residues selected from 22P/27P/437H/466I, 22P/34Y/278S/318K/437H/466I, 22P/466I, 41T/42T/43-/397S, 43T/244G/397S, 45T/397S, and 397S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280,

or amino acid residues selected from F22P/Q27P/T437H/M466I, F22P/D34Y/K278S/R318K/T437H/M466I, F22P/M466I, I41T/K42T/F43-/K397S, F43T/V244G/K397S, E45T/K397S, and K397S.

[0225] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280 at amino acid positions 173, 175, 176, 179, 182, 196, 197, 208, 263, 273, 274, 277, 308, 310, 319, 328, 341, 376, 388, 405, 408, 411, 462, 477, or 480. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280, or amino acid residues selected from 173P, 175L, 175R, 176H, 179C, 179K, 179R, 182I, 196D, 197L, 208T, 263R, 263T, 273M, 274L, 277D, 308Y, 310S, 319A, 328A, 341T, 376T, 388V, 405L, 408G, 411T, 462Y, 477L, 477W, and 480K. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280, or amino acid residues selected from T173P, S175L, S175R, N176H, Q179C, Q179K, Q179R, T182I, G196D, M197L, Q208T, S263R, S263T, K273M, P274L, S277D, H308Y, E310S, V319A, V328A, V341T, E376T, L388V, Q405L, A408G, G411T, L462Y, Q477L, Q477W, and R480K.

[0226] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280 at amino acid positions 195, 203, 273, 274, 277, 278, 291, 299, 300, 324, or 477. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or more residue differences as compared to SEQ ID NO: 2280, or amino acid residues selected from 195T, 203Q, 273E, 273G, 273M, 273N, 273Q, 274L, 277D, 278D, 291L, 299G, 299H, 300S, 324T, and 477W. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2280, or to a reference sequence corresponding to SEQ ID NO: 2280, and one or

more residue differences as compared to SEQ ID NO: 2280, or amino acid residues selected from K195T, M203Q, K273E, K273G, K273M, K273N, K273Q, P274L, S277D, K278D, R291L, S299G, S299H, K300S, I324T, and Q477W.

[0227] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2342, or to a reference sequence corresponding to SEQ ID NO: 2342, and one or more residue differences as compared to SEQ ID NO: 2342 at amino acid positions 34/273/300/477/480, 34/396, or 300/396/477/480. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2342, or to a reference sequence corresponding to SEQ ID NO: 2342, and one or more residue differences as compared to SEQ ID NO: 2342, or amino acid residues selected from 34D/273M/300S/477L/480K, 34D/396T, and 300S/396T/477L/480K. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2342, or to a reference sequence corresponding to SEQ ID NO: 2342, and one or more residue differences as compared to SEQ ID NO: 2342, or amino acid residues selected from Y34D/K273M/K300S/Q477L/R480K, Y34D/S396T, and K300S/S396T/Q477L/R480K.

[0228] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438 at amino acid positions 27/34/173/176/179/195/277, 27/173, 27/173/176, 27/173/176/179/273/277, 27/173/176/179/277, 27/173/179/273, 27/173/179/273/277, 27/173/179/277, 27/176/179/273, 27/179, 27/179/273/277, 27/179/277, 34, 34/173/176/197/277, 105/173/176/197/277, 173/176/179, 173/176/179/197, 173/176/179/277, 173/179, 173/179/273, 173/179/273/277, 173/277, 176/179, 176/179/197, 176/179/273, 176/179/273/277, 179, 179/197, 179/273, or 179/273/277. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438, or amino acid residues selected from 27P/34D/173P/176H/179K/195T/277D, 27P/173P, 27P/173P/176H, 27P/173P/176H/179K/273M/277D, 27P/173P/176H/179R/277D, 27P/173P/179K/273M, 27P/173P/179K/273M/277D, 27P/173P/179K/277D, 27P/176H/179R/273M, 27P/179K, 27P/179K/273M/277D, 27P/179R/277D, 34D, 34D/173P/176H/197L/277D, 105G/173P/176H/197L/277D,

173P/176H/179K/277D, 173P/176H/179R, 173P/176H/179R/197L, 173P/179K/273M/277D, 173P/179R, 173P/179R/273M, 173P/179R/273M/277D, 173P/277D, 176H/179K/273M, 176H/179K/273M/277D, 176H/179R, 176H/179R/197L, 176H/179R/273M/277D, 179K, 179K/197L, 179K/273M, 179K/273M/277D, 179R, and 179R/197L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438, or amino acid residues selected from Q27P/Y34D/T173P/N176H/Q179K/K195T/S277D, Q27P/T173P, Q27P/T173P/N176H, Q27P/T173P/N176H/Q179K/K273M/S277D, Q27P/T173P/N176H/Q179R/S277D, Q27P/T173P/Q179K/K273M, Q27P/T173P/Q179K/K273M/S277D, Q27P/T173P/Q179K/S277D, Q27P/N176H/Q179R/K273M, Q27P/Q179K, Q27P/Q179K/K273M/S277D, Q27P/Q179R/S277D, Y34D, Y34D/T173P/N176H/M197L/S277D, E105G/T173P/N176H/M197L/S277D, T173P/N176H/Q179K/S277D, T173P/N176H/Q179R, T173P/N176H/Q179R/M197L, T173P/Q179K/K273M/S277D, T173P/Q179R, T173P/Q179R/K273M, T173P/Q179R/K273M/S277D, T173P/S277D, N176H/Q179K/K273M, N176H/Q179K/K273M/S277D, N176H/Q179R, N176H/Q179R/M197L, N176H/Q179R/K273M/S277D, Q179K, Q179K/M197L, Q179K/K273M, Q179K/K273M/S277D, Q179R, and Q179R/M197L.

[0229] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438 at amino acid positions 177, 199, 202, 229, 292, 339, 342, 352, 363, 376, 406, 407, 430, 487, 508, or 525. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438, or amino acid residues selected from 177C, 199E, 202V, 229L, 292A, 339T, 342T, 352A, 352T, 363G, 376H, 406D, 406P, 407N, 430G, 430S, 487R, 508C, and 525T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2438, or to a reference sequence corresponding to SEQ ID NO: 2438, and one or more residue differences as compared to SEQ ID NO: 2438, or amino acid residues selected from R177C, D199E, L202V, W229L, S292A, V339T, E342T, P352A, P352T, P363G, E376H, G406D, G406P, P407N, K430G, K430S, K487R, K508C, and V525T.

[0230] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino

acid residues 12 to carboxy terminal of SEQ ID NO: 2452, or to a reference sequence corresponding to SEQ ID NO: 2452, and one or more residue differences as compared to SEQ ID NO: 2452 at amino acid positions 176/183, 176/183/205, 183, 183/205, 183/205/408, or 205. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2452, or to a reference sequence corresponding to SEQ ID NO: 2452, and one or more residue differences as compared to SEQ ID NO: 2452, or amino acid residues selected from 176N/183R, 176N/183R/205L, 183G, 183G/205L, 183R, 183R/205L, 183R/205L/408V, and 205L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2452, or to a reference sequence corresponding to SEQ ID NO: 2452, and one or more residue differences as compared to SEQ ID NO: 2452, or amino acid residues selected from H176N/D183R, H176N/D183R/R205L, D183G, D183G/R205L, D183R, D183R/R205L, D183R/R205L/A408V, and R205L.

[0231] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562 at amino acid positions 177, 177/292/339/342/406, 177/292/339/406, 177/292/342/363/376/406, 177/292/342/406, 177/339/342, 177/339/342/376, 177/339/342/406, 177/339/342/406/487, 177/342/376/407, 177/342/406/430, 177/407, 177/487, 292/339/342/376/406/487, 292/342, 292/342/406, 292/406, 339/342, 339/342/407, 339/342/407/487, or 406. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562, or amino acid residues selected from 177K, 177K/292A/339T/342T/406D, 177K/292A/339T/406D, 177K/292A/339T/406P, 177K/292A/342T/363G/376H/406D, 177K/292A/342T/406P, 177K/339T/342T, 177K/339T/342T/376H, 177K/339T/342T/406D, 177K/339T/342T/406P/487R, 177K/342T/376H/407N, 177K/342T/406P/430G, 177K/407N, 177K/487R, 292A/339T/342T/376H/406P/487R, 292A/342T, 292A/342T/406D, 292A/406P, 339T/342T, 339T/342T/407N, 339T/342T/407N/487R, and 406P. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562, or amino acid residues selected from R177K,

R177K/S292A/V339T/E342T/G406D, R177K/S292A/V339T/G406D, R177K/S292A/V339T/G406P, R177K/S292A/E342T/P363G/E376H/G406D, R177K/S292A/E342T/G406P, R177K/V339T/E342T, R177K/V339T/E342T/E376H, R177K/V339T/E342T/G406D, R177K/V339T/E342T/G406P/K487R, R177K/E342T/E376H/P407N, R177K/E342T/G406P/K430G, R177K/P407N, R177K/K487R, S292A/V339T/E342T/E376H/G406P/K487R, S292A/E342T, S292A/E342T/G406D, S292A/G406P, V339T/E342T, V339T/E342T/P407N, V339T/E342T/P407N/K487R, and G406P.

[0232] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562 at amino acid positions 174, 187, 190, 198, 267, 274, 277, 307, 316, 343, 359, 379, 402/407, 407, 409, 412, 414, 481, 494, or 523. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562, or amino acid residues selected from 174I, 187T, 190G, 198P, 198S, 198T, 267G, 274V, 277S, 307K, 316L, 343R, 359F, 379M, 402P/407A, 407S, 409S, 412P, 414Q, 481H, 494T, 494Y, 523A, and 523H. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2562, or to a reference sequence corresponding to SEQ ID NO: 2562, and one or more residue differences as compared to SEQ ID NO: 2562, or amino acid residues selected from W174I, Y187T, T190G, D198P, D198S, D198T, S267G, P274V, D277S, T307K, V316L, L343R, L359F, L379M, S402P/P407A, P407S, D409S, D412P, E414Q, D481H, H494T, H494Y, E523A, and E523H.

[0233] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576 at amino acid positions 202, 208, 221, 224, 235, 236, 238, 239, 242, 249, 251, 252, 259, 263, 405/406, 406, 477, 484/487, or 487. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from 202M, 208M, 221I, 224V, 235R, 236R, 238I, 238L, 239G, 239L, 239R, 239S, 242L, 249L, 249Q, 251A, 252R, 259F, 263R, 405G/406G, 405V/406G, 406E, 406M, 406T, 477I, 484L/487K, 484Y/487K,

and 487K. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from L202M, Q208M, L221I, T224V, K235R, G236R, M238I, M238L, E239G, E239L, E239R, E239S, I242L, E249L, E249Q, Q251A, A252R, Y259F, S263R, Q405G/P406G, Q405V/P406G, P406E, P406M, P406T, L477I, R484L/R487K, R484Y/R487K, and R487K.

[0234] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576 at amino acid positions 174/190, 174/190/202/204/254, 174/190/202/254, 174/190/204, 174/190/236, 174/202/204/254, 174/202/204/254/353, 174/204/236/254/353, 187, 187/224, 187/224/321/436, 187/224/379/436, 187/249, 187/249/321/379/436, 204/254/353, 249/379, 359/407, 359/407/412, 379, or 436. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from 174I/190G, 174I/190G/202H/204M/254I, 174I/190G/202H/254I, 174I/190G/204M, 174I/190G/236R, 174I/202H/204M/254I, 174I/202H/204M/254I/353V, 174I/204M/236R/254I/353V, 187T, 187T/224V, 187T/224V/321R/436S, 187T/224V/379M/436S, 187T/249M, 187T/249M/321R/379M/436S, 204V/254I/353V, 249M/379M, 359F/407A, 359F/407S/412P, 379M, and 436S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from W174I/T190G, W174I/T190G/L202H/F204M/R254I, W174I/T190G/L202H/R254I, W174I/T190G/F204M, W174I/T190G/G236R, W174I/L202H/F204M/R254I, W174I/L202H/F204M/R254I/R353V, W174I/F204M/G236R/R254I/R353V, Y187T, Y187T/T224V, Y187T/T224V/A321R/P436S, Y187T/T224V/L379M/P436S, Y187T/E249M, Y187T/E249M/A321R/L379M/P436S, F204V/R254I/R353V, E249M/L379M, L359F/P407A, L359F/P407S/D412P, L379M, and P436S.

[0235] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ

ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576 at amino acid positions 219, 293, 300, 310, 313, 318, 340, 364, or 392. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from 219Q, 293R, 300R, 310D, 313Q, 318P, 340L, 340T, 364S, 392Q, and 392S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from R219Q, S293R, S300R, E310D, R313Q, K318P, I340L, I340T, P364S, K392Q, and K392S.

[0236] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576 at amino acid positions 160, 161, 173, 175, 222, 244, 278, 293, 300, 301, 310, 318, 319, 324, 334, 339, 341, 342, 366, 367, 368, 370, 376, or 378. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from 160A, 160D, 160T, 160W, 161A, 161L, 173H, 175H, 175P, 222N, 244I, 244S, 278A, 278L, 293R, 300R, 301F, 310D, 318P, 318R, 318S, 319P, 324A, 334S, 339A, 339S, 341L, 342S, 366F, 366H, 366P, 367V, 368R, 370L, 370R, 376A, 376G, 376R, 376S, 376V, and 378M. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2576, or to a reference sequence corresponding to SEQ ID NO: 2576, and one or more residue differences as compared to SEQ ID NO: 2576, or amino acid residues selected from M160A, M160D, M160T, M160W, K161A, K161L, P173H, S175H, S175P, A222N, V244I, V244S, S278A, S278L, S293R, S300R, W301F, E310D, K318P, K318R, K318S, V319P, I324A, T334S, T339A, T339S, V341L, T342S, A366F, A366H, A366P, Q367V, S368R, Q370L, Q370R, E376A, E376G, E376R, E376S, E376V, and V378M.

[0237] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2714, or to a reference sequence corresponding to SEQ ID NO: 2714, and one or more residue differences as compared to SEQ ID NO: 2714 at amino acid positions 187, 187/224, 187/224/263, 187/224/407, 187/224/407/412, 187/224/412, 187/254/263/359/379/407, 187/263, 187/359, 187/379, 224/359/407, 249/263, 249/263/407, 263, or 263/407/412. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2714, or to a reference sequence corresponding to SEQ ID NO: 2714, and one or more residue differences as compared to SEQ ID NO: 2714, or amino acid residues selected from 187T, 187T/224V, 187T/224V/263R, 187T/224V/407A, 187T/224V/407A/412P, 187T/224V/412P, 187T/254R/263R/359F/379M/407S, 187T/263R, 187T/359F, 187T/379M, 224V/359F/407A, 249M/263R, 249M/263R/407S, 263R, and 263R/407S/412P. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2714, or to a reference sequence corresponding to SEQ ID NO: 2714, and one or more residue differences as compared to SEQ ID NO: 2714, or amino acid residues selected from Y187T, Y187T/T224V, Y187T/T224V/S263R, Y187T/T224V/P407A, Y187T/T224V/P407A/D412P, Y187T/T224V/D412P, Y187T/I254R/S263R/L359F/L379M/P407S, Y187T/S263R, Y187T/L359F, Y187T/L379M, T224V/L359F/P407A, E249M/S263R, E249M/S263R/P407S, S263R, and S263R/P407S/D412P.

[0238] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2876, or to a reference sequence corresponding to SEQ ID NO: 2876, and one or more residue differences as compared to SEQ ID NO: 2876 at amino acid positions 160/263/318/339, 175/244/293/364/407, 219/310, 219/310/340, 244/293/313, 244/293/334, 244/313, 244/313/334/364/407, 263/310/318/366, 263/318, 293/313/334/364, 293/334/364/407, 310/318/340, 318, or 334/364. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2876, or to a reference sequence corresponding to SEQ ID NO: 2876, and one or more residue differences as compared to SEQ ID NO: 2876, or amino acid residues selected from 60T/263R/318P/339S, 175H/244I/293R/364S/407P, 219S/310D, 219S/310D/340T, 244I/293R/313Q, 244I/313Q, 244I/313Q/334S/364S/407S, 244S/293R/334S, 263R/310D/318S/366H, 263R/318P, 293R/313Q/334S/364S, 293R/334S/364S/407P, 310D/318P/340T, 318P, and 334S/364S. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12

to carboxy terminal of SEQ ID NO: 2876, or to a reference sequence corresponding to SEQ ID NO: 2876, and one or more residue differences as compared to SEQ ID NO: 2876, or amino acid residues selected from M160T/S263R/K318P/T339S, S175H/V244I/S293R/P364S/A407P, R219S/E310D, R219S/E310D/I340T, V244I/S293R/R313Q, V244I/R313Q, V244I/R313Q/T334S/P364S/A407S, V244S/S293R/T334S, S263R/E310D/K318S/A366H, S263R/K318P, S293R/R313Q/T334S/P364S, S293R/T334S/P364S/A407P, E310D/K318P/I340T, K318P, or T334S/P364S.

[0239] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884 at amino acid positions 254/293/310, 254/293/310/334/340/407, 310/334, 334, 334/340, 334/407, 340, or 407. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884, or amino acid residues selected from 254R/293R/310D, 254R/293R/310D/334S/340T/407P, 310D/334S, 334S, 334S/340T, 334S/407P, 340T, and 407P. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884, or amino acid residues selected from I254R/S293R/E310D, I254R/S293R/E310D/T334S/I340T/A407P, E310D/T334S, T334S, T334S/I340T, T334S/A407P, I340T, and A407P.

[0240] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884 at amino acid positions 183, 190, 203, 232, 252, 259/263, 261/263, 397, 408, or 413. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884, or amino acid residues selected from 183V, 190N, 203L, 232G, 252H, 259F/263S, 261S/263S, 397Q, 408D, and 413T. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more

sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884, or amino acid residues selected from D183V, T190N, M203L, D232G, A252H, Y259F/R263S, V261S/R263S, K397Q, A408D, and G413T.

[0241] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884 at amino acid positions 179, 200, 201, 208, 218, 223, 227, 229, 230, 235, 297, or 408. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2884, or amino acid residues selected from 179V, 200Q, 201R, 208G, 218I, 223L, 227G, 227S, 229G, 229S, 230V, 235N, 297Q, and 408D. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2884, or to a reference sequence corresponding to SEQ ID NO: 2884, and one or more residue differences as compared to SEQ ID NO: 2952, or amino acid residues selected from K179V, K200Q, A201R, Q208G, V218I, D223L, L227G, L227S, W229G, W229S, I230V, K235N, S297Q, and A408D.

[0242] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2910, or to a reference sequence corresponding to SEQ ID NO: 2910, and one or more residue differences as compared to SEQ ID NO: 2910 at amino acid positions 307/429/474, or 508. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2910, or to a reference sequence corresponding to SEQ ID NO: 2910, and one or more residue differences as compared to SEQ ID NO: 2910, or amino acid residues selected from 307K/429A/474L and 508Q. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2910, or to a reference sequence corresponding to SEQ ID NO: 2910, and one or more residue

differences as compared to SEQ ID NO: 2910, or amino acid residues selected from T307K/R429A/M474L and K508Q.

[0243] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2974, or to a reference sequence corresponding to SEQ ID NO: 2974, and one or more residue differences as compared to SEQ ID NO: 2974 at amino acid positions 190, 190/203/205/508, 205/208, 205/223, 205/230, 205/239/403/508, 205/297/508, 205/370/403, 205/403, 205/413, 205/508, or 218/319/508. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2974, or to a reference sequence corresponding to SEQ ID NO: 2974, and one or more residue differences as compared to SEQ ID NO: 2974, or amino acid residues selected from 190N, 190N/203F/205R/508K, 205R/208G, 205R/223L, 205R/230V, 205R/239S/403F/508K, 205R/297Q/508K, 205R/370R/403F, 205R/403F, 205R/413T, 205R/508K, and 218I/319M/508K. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2974, or to a reference sequence corresponding to SEQ ID NO: 2974, and one or more residue differences as compared to SEQ ID NO: 2974, or amino acid residues selected from T190N, T190N/M203F/L205R/Q508K, L205R/Q208G, L205R/D223L, L205R/I230V, L205R/E239S/L403F/Q508K, L205R/S297Q/Q508K, L205R/Q370R/L403F, L205R/L403F, L205R/G413T, L205R/Q508K, and V218I/V319M/Q508K.

[0244] In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2998, or to a reference sequence corresponding to SEQ ID NO: 2998, and one or more residue differences as compared to SEQ ID NO: 2998 at amino acid positions 230/251/352/357, 238/297/403, 238/297/403/466, 297, and 403/466. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2998, or to a reference sequence corresponding to SEQ ID NO: 2998, and one or more residue differences as compared to SEQ ID NO: 2998, or amino acid residues selected from 230I/251A/352T/357I, 238L/297V/403F, 238L/297V/403F/466L, 297V, and 403F/466L. In some embodiments, the engineered TnT polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to carboxy terminal of SEQ ID NO: 2998, or to a reference sequence corresponding to SEQ ID NO: 2998, and one or more residue

differences as compared to SEQ ID NO: 2998, or amino acid residues selected from V230I/Q251A/P352T/V357I, M238L/S297V/L403F, M238L/S297V/L403F/I466L, S297V, and L403F/I466L.

[0245] As will be appreciated by the skilled artisan, in some embodiments, one or a combination of residue differences above that is selected can be kept constant (i.e., maintained) in the engineered TnT as a core feature, and additional residue differences at other residue positions incorporated into the sequence to generate additional engineered TnT polypeptides with improved properties. Accordingly, it is to be understood for any engineered TnT containing one or a subset of the residue differences above, the present invention contemplates other engineered TnTs that comprise the one or subset of the residue differences, and additionally one or more residue differences at the other residue positions disclosed herein.

[0246] As noted above, the engineered TnT polypeptides are also capable of converting substrates (e.g., NTP-3'-O-RBG or a natural or modified NTP and an oligo acceptor substrate) to products (e.g., an oligo acceptor substrate with an added nucleotide-3'-O-RBG). In some embodiments, the engineered TnT polypeptide is capable of converting the substrate compounds to the product compound with at least 1.1, 1.2 fold, 1.5 fold, 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold, or more activity relative to the activity of the reference polypeptide of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0247] In some embodiments, the engineered TnT capable of converting the substrate compounds to the product compounds with at least 1.2 fold or at least 2 fold the activity relative to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, comprises an amino acid sequence selected from the even-numbered sequences in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0248] In some embodiments, the engineered TnT capable of converting an oligo acceptor substrate of 4 to 7 basepairs to the oligo acceptor substrate extended by one nucleotide with at least 1.3 fold, 1.4 fold, or 1.5 fold the activity relative to SEQ ID NO: 148 or 256, comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 682.

[0249] In some embodiments, the engineered TnT capable of converting an oligo acceptor substrate of 4 to 7 basepairs to the oligo acceptor substrate extended by one nucleotide, has activity in the conversion of a variety of oligo acceptor substrates and comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 148, 256, or 682.

[0250] In some embodiments, the engineered TnT capable of converting an oligo acceptor substrate of 3 to 4 basepairs to the oligo acceptor substrate extended by one nucleotide with at least 31% or 91% conversion comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 932.

[0251] In some embodiments, the engineered TnT capable of converting an oligo acceptor substrate to the oligo acceptor substrate extended by a glycol nucleic acid nucleotide ((S)-GNA) or a locked nucleic acid nucleotide (LNA) with at least 75% conversion comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 992.

[0252] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NO: 2, that increases substrate promiscuity for NTP-3'-O-RBG substrates of 2'-methoxyadenosine, 2'-methoxycytidine, 2'-methoxyguanosine, 2'-methoxyuridine, 2'-fluoroadenosine, 2'-fluorocytidine, 2'-fluoroguanosine, 2'-fluorouridine, adenosine ribonucleotide, cytidine ribonucleotide, guanosine ribonucleotide, uridine ribonucleotide, phosphorothioate linkage, 5'-phosphorylation modification, adenosine (S)-glycol nucleic acid, cytidine (S)-glycol nucleic acid, guanosine (S)-glycol nucleic acid, thymidine (S)-glycol nucleic acid, uridine (S)-glycol nucleic acid, C2'-endo locked adenosine ribonucleotide, C2'-endo locked cytidine ribonucleotide, C2'-endo locked guanosine ribonucleotide, C2'-endo locked thymidine ribonucleotide, C2'-endo locked uridine ribonucleotide, 2'-O-(2-methoxyethyl)adenosine, 2'-O-(2-methoxyethyl)cytidine, 2'-O-(2-methoxyethyl)guanosine, 2'-O-(2-methoxyethyl)uridine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxythymidine, 2',3'-dideoxyuridine and vinylphosphonate 2'O-methoxy uridine, 1-((2S))-1-(2,3-dihydroxypropyl))thymine, 2'O-hexadecyl cytidine, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0253] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NO: 2, that increases substrate promiscuity for NTP-3'-O-RBG substrates of 2'-methoxyadenosine, 2'-methoxycytidine, 2'-methoxyguanosine, 2'-methoxyuridine, 2'-fluoroadenosine, 2'-fluorocytidine, 2'-fluoroguanosine, 2'-fluorouridine, adenosine ribonucleotide, cytidine ribonucleotide, guanosine ribonucleotide, uridine ribonucleotide, phosphorothioate linkage, 5'-phosphorylation modification, adenosine (S)-glycol nucleic acid, cytidine (S)-glycol nucleic acid, guanosine (S)-glycol nucleic acid, thymidine (S)-glycol nucleic acid, uridine (S)-glycol nucleic acid, C2'-endo locked adenosine ribonucleotide, C2'-endo locked cytidine ribonucleotide, C2'-endo locked guanosine ribonucleotide, C2'-endo locked thymidine ribonucleotide, C2'-endo locked uridine ribonucleotide, 2'-O-(2-methoxyethyl)adenosine, 2'-O-(2-methoxyethyl)cytidine, 2'-O-(2-methoxyethyl)guanosine, 2'-O-(2-methoxyethyl)uridine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxythymidine, 2',3'-dideoxyuridine and vinylphosphonate 2'O-methoxy uridine, 1-((2S))-1-(2,3-dihydroxypropyl))thymine, 2'O-hexadecyl cytidine, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 992.

[0254] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NO: 2, that increases substrate promiscuity for oligo acceptor substrates of 5'-6-FAM-T10mCmCmUfA, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T36fCmAmUmC,

5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T41*fC*mAfA, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56mA*fC*mAfA, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T51fGmUfGmU, 5'-6-FAM-T11AmC*mA*mG, 5'-6-FAM-T15mAmUmCmU, 5'-6-FAM-T46mUmCmUmU, 5'-6-FAM-T41mAmUmCmU, T8mUfGmUfC, T8fCmAmUmC, T8mCfCmCfG, T8mCfCmGfG, mAmAmAmAmUmCmU, mAmAmAmUmCmU, mAmAmUmCmU, mAmUmCmU, mUmCmU, 5'P-mUmCmU, mC*mA*mGmA, mAfGmUfG, mGmUmC*mC, 5'-6-FAM-T9mAmAmAmUmCmU, 5'-6-FAM-T9mAmAmAmUmCmU(sgna)A, 5'-6-FAM-T9mAmAmAmUmCmU+A, 5'-6-FAM-T9mAmAmAmUmCmU+G, 5'-6-FAM-T9mAmAmAmUmCmU+C, 5'-6-FAM-T9mAmAmAmUmCmU+U, 5'-6-FAM-T9mAmAmAmUmCmU+A+A, 5'-6-FAM-T9mAmAmAmUmCmU+G+G, 5'-6-FAM-T9mAmAmAmUmCmU+U+U, 5'-6-FAM-T16mAfGmAfG, 5'-6-FAM-T26fCfAfUmG, 5'-6-FAM-T10AmC*mA*mG, 5'-6-FAM-T16*fC*mAfAfA, 5'-6-FAM-T16*fA*mAmGfAfG, 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T21mUmAmG*mA, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T25mAmGfUfGfG, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T31mUfCmAmU, 5'-6-FAM-T35fAfGfUmGfG, 5'-6-FAM-T51mAmUmCfC, 5'-6-FAM-T56fGmUfCmU, T8AmC*mA*mG, T8mCfCmGfG, T8*fC*mAfAfA, T8*fA*mAmGfAfG, T8mGfUfAfC, T8fUfUfUfC, T8mAfUfCfC, T8mAmUfUmGfG, T8mAmGfUfGfG, T8mAmGfAmGfG, T8*fU*fUfAfU, T8mGfUmGfG, T8fAmUmUfG, T8mU*fA*fAfG, T8fAfGfUmGfG, T8fGmUfCmU, T8mA*fC*mAfA, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0255] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases soluble expression or isolated protein yield of the engineered TnT in a bacterial host cell, particularly in *E. coli*, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0256] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases thermostability of the engineered TnT, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0257] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases the

activity of the engineered TnT at high temperatures (by way of example and not limitation, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 68 °C, 68.5 °C, 69 °C, 70 °C, or 72 °C), as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0258] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that reduces the by-product formation of the engineered TnT, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0259] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases specific activity of the engineered TnT on one or more NTP-3'-O-RBG or a natural or modified NTP substrates, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0260] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases specific activity of the engineered TnT on one or more oligo acceptor substrates, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0261] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases specific activity of the engineered TnT on one or more oligo acceptor substrates comprising a length of three to seven nucleotides, as compared to a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0262] In some embodiments, the engineered TnT with an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that increases incorporation efficiency in extension of an oligo acceptor substrate by addition of an NTP or NQP of greater than 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. when

compared to the incorporation efficiency of a wild-type or engineered reference TnT, comprises an amino acid sequence selected from the even-numbered sequences of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0263] In some embodiments, the engineered TnT with improved properties has an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and at least one substitution or substitution set at amino acid positions selected from 22, 34, 60, 160, 162, 173, 174, 176, 177, 179, 187, 190, 191, 193, 194, 197, 203, 204, 205, 208, 222, 224, 230, 231, 236, 246, 251, 254, 260, 261, 262, 263, 264, 267, 273, 274, 277, 278, 295, 296, 297, 299, 300, 308, 310, 313, 318, 319, 325, 329, 334, 335, 339, 342, 343, 352, 353, 357, 361, 363, 367, 368, 376, 379, 381, 390, 396, 397, 402, 404, 405, 406, 407, 408, 409, 412, 416, 417, 420, 433, 437, 444, 451, 460, 462, 465, 466, 477, 480, 485, 487, 490, 492, 495, 503, 504, 508, and 525, or any combinations thereof, wherein the amino acid positions are numbered with reference to amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or with reference to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, .

[0264] In some embodiments, the engineered TnT with improved properties has an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and at least one substitution or substitution set at amino acid positions selected from 22P, 34Y, 60H, 160T, 162P, 173P, 174I/V/W, 176H, 177K, 179K, 187T/Y, 190R/T, 191F, 193S/T, 194L, 197M, 203M, 204M, 205L, 208E/G/Q, 222A, 224V, 230V, 231M/N, 236R, 246V, 251A, 254I/R, 260R, 261V, 262M, 263R/S, 264L, 267S, 273I/K/R/M, 274P, 277S, 278S, 295D, 296V, 297S, 299S, 300K/S, 308H, 310D, 313R, 318K/P/Q, 319T/V, 325V, 329L, 334S, 335V, 339S/T/V, 342T, 343L, 352T, 353L/R/V, 357I, 361V, 363P, 367Q, 368S, 376E, 379L, 381Y, 390M/S, 396T, 397K, 402S, 404S, 405Q, 406P, 407A/P, 408V, 409E, 412P, 416A, 417A, 420V, 433P, 437H, 444G, 451R, 460Q, 462L/M, 465G, 466I, 477A/L/Q, 480K, 485I, 487R,

490V, 492H/M, 495G, 503L, 504S, 508Q, and 525V, or any combinations thereof, wherein the amino acid positions are numbered with reference to amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or with reference to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0265] In some embodiments, the engineered TnT with improved properties has an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and at least one substitution or substitution set at amino acid positions selected from F22P, D34Y, R60H, M160T, T162P, T173P, L174I/V/W, N176H, R177K, Q179K, I187T/Y, E/R190R/T, N191F, V193S/T, H194L, N197M, E203M, F204M, R205L, I208E/G/Q, K222A, T224V, I230V, S231M/N, G236R, E246V, Q251A, L/R254I/R, Q260R, S261V, F262M, H263R/S, A264L, R267S, V273I/K/R/M, G274P, D277S, K278S, T295D, L296V, K297S, T299S, T300K/S, Y308H, E310D, A313R, R318K/P/Q, A/T319T/V, W325V, Q329L, T334S, F335V, A339S/T/V, E342T, I343L, P352T, Q353L/R/V, V357I, I361V, S363P, E367Q, K368S, Q376E, M379L, L381Y, C390M/S, S396T, Q397K, G402S, P404S, S405Q, G406P, K/P407A/P, A408V, D409E, D412P, S416A, C417A, I420V, H433P, T437H, S444G, K451R, G460Q, F/M462L/M, A465G, M466I, R477A/L/Q, R480K, E485I, K487R, M490V, D/M492H/M, S495G, I503L, Q504S, K508Q, and F525V, or any combinations thereof, wherein the amino acid positions are numbered with reference to amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or with reference to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0266] In some embodiments, the engineered TnT with improved properties has an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478,

668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and at least one substitution at amino acid position selected from 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, and 526, or any combinations thereof, wherein the amino acid positions are numbered with reference to amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or with reference to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0267] In some embodiments, the engineered TnT with improved properties has an amino acid sequence comprising amino acid residues 12 to carboxy terminus of an even-numbered SEQ ID NO. of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008, or an amino acid sequence comprising an even-numbered SEQ ID NO. of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0268] In some embodiments, the engineered TnT with improved properties has an amino acid sequence comprising amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or comprising SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0269] In some embodiments, the engineered TnT, comprises an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to one of the sequences of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 32282714, 2876, 2884, 2910, 2974, 2998, and 3002, as provided in the Examples.

[0270] In addition to the residue positions specified above, any of the engineered TnT polypeptides disclosed herein can further comprise other residue differences relative to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2754, 2944, 2952, 2978, 3042, 3066, 3126, 3202, and 3228, at other residue positions (i.e., residue positions other than those included herein). Residue differences at these other residue positions can provide for additional variations in the amino acid sequence without adversely affecting the ability of the polypeptide to carry out the conversion of substrate to product. Accordingly, in some embodiments, in addition to the amino acid residue differences present in any one of the engineered TnTs polypeptides selected from the even-numbered sequences in the range of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008, the sequence can further comprise 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-14, 1-15, 1-16, 1-18, 1-20, 1-22, 1-24, 1-26, 1-30, 1-35, 1-40, 1-45, 1-50, 1-100, or 1-150 residue differences at other amino acid residue positions as compared to the SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002. In some embodiments, the number of amino acid residue differences as compared to the reference sequence can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 30, 35, 40, 45, 50, 100, or 150 residue positions. In some embodiments, the number of amino acid residue differences as compared to the reference sequence can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, or 25 residue positions. The residue differences at these other positions can be conservative changes or non-conservative changes. In some embodiments, the residue differences can comprise conservative substitutions and non-conservative substitutions as compared to the TnT polypeptide of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0271] In some embodiments, the present invention also provides engineered polypeptides that comprise a fragment of any of the engineered TnT polypeptides described herein that retains the functional activity and/or improved property of that engineered TnT. Accordingly, in some embodiments, the present invention provides a polypeptide fragment capable of converting substrate to product under suitable reaction conditions, wherein the fragment comprises at least about 90%, 95%, 96%, 97%, 98%, or 99% of a full-length or truncated amino acid sequence of an engineered TnT of the present invention, such as an exemplary TnT polypeptide selected from the even-numbered sequences in the range of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008. In some embodiments, the engineered TnT can have an amino acid sequence comprising a deletion in any one of the TnT polypeptide sequences described herein, such as the exemplary engineered polypeptides of the even-numbered sequences in the range of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0272] Thus, for each and every embodiment of the engineered TnT polypeptides of the invention, the amino acid sequence can comprise deletions of one or more amino acids, 2 or more amino acids, 3 or more amino acids, 4 or more amino acids, 5 or more amino acids, 6 or more amino acids, 8 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, up to 20% of the total number of amino acids, or up to 30% of the total number of amino acids of

the TnT polypeptides, where the associated functional activity and/or improved properties of the engineered TnT described herein are maintained. In some embodiments, the deletions can comprise 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, or 1-50 amino acid residues. In some embodiments, the number of deletions can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 30, 35, 40, 45, or 50 amino acid residues. In some embodiments, the deletions can comprise deletions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, or 25 amino acid residues.

[0273] In some embodiments, the engineered TnT polypeptide herein can have an amino acid sequence comprising an insertion as compared to any one of the engineered TnT polypeptides described herein, such as the exemplary engineered polypeptides of the even-numbered sequences in the range of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008. Thus, for each and every embodiment of the TnT polypeptides of the invention, the insertions can comprise one or more amino acids, 2 or more amino acids, 3 or more amino acids, 4 or more amino acids, 5 or more amino acids, 6 or more amino acids, 8 or more amino acids, 10 or more amino acids, 15 or more amino acids, 20 or more amino acids, 30 or more amino acids, 40 or more amino acids, or 50 or more amino acids, where the associated functional activity and/or improved properties of the engineered TnT described herein is maintained. The insertions can be to amino or carboxy terminus, or internal portions of the TnT polypeptide.

[0274] In some embodiments, the engineered TnT described herein can have an amino acid sequence comprising a sequence selected from the even-numbered sequences in the range of SEQ ID NO: 2-1056, 1130-1880, and 1928-3008, and optionally one or several (e.g., up to 3, 4, 5, or up to 10) amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-75, 1-100, or 1-150 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally around 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the substitutions can be conservative or non-conservative substitutions.

[0275] In the above embodiments, the suitable reaction conditions for the engineered polypeptides are provided in **Tables 5.1**, **6.1**, **7.1**, **8.1**, **9.1**, **10.1**, **11.1**, **12.1**, **13.1**, **14.1**, **15.1**, **16.1**, **17.1**, **18.1**, **19.1**, **20.1**, **21.1**, **22.1**, **23.1**, **24.1**, **25.1**, **26.1**, **27.1**, **28.1**, **29.1**, **30.1**, **31.1**, **32.1**, **35.1**, **36.1**, **37.1**, **38.1**, **39.1**, **40.1**, **41.1**, **42.1**, **43.1**, **44.1**, **45.1**, **46.1**, **47.1**, **48.1**, **49.1**, **50.1**, **51.1**, **52.1**, **53.1**, **54.1**, **56.1**, **57.1**, **58.1**, **59.1**, **60.1**, **61.1**, **62.1**, **63.1**, **64.1**, **65.1**, **66.1**, **67.1**, **68.1**, **69.1**, **70.1**, **71.1**, **72.1**, **73.1**, **74.1**, **75.1**, **76.1**, **77.1**, **78.1**, **79.1**, **80.1**, **81.1**, **82.1**, **83.1**, **84.1**, **85.1**, **86.1**, **87.1**, **88.1**, **89.1**, and **90.1**, ,as described in the Examples herein.

[0276] In some embodiments, the polypeptides of the present invention are fusion polypeptides in which the engineered polypeptides are fused to other polypeptides, such as, by way of example and not limitation, antibody tags (e.g., myc epitope), purification sequences (e.g., His tags for binding to metals), cell localization signals (e.g., secretion signals), and polypeptides with enzymatic activity. Thus, the engineered polypeptides described herein can be used with or without fusions to other polypeptides.

[0277] In one embodiment of the engineered TnT polypeptides of the present invention, the polypeptide further comprises an N-terminal truncation of 1-156 amino acids of the polypeptide sequence relative to any even-numbered sequence set forth in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008.

[0278] In some embodiments, the engineered TnT polypeptides of the invention can be fused to another second polypeptide, such as a polypeptide with a different enzymatic activity. In some embodiments, the present provides a fusion polypeptide comprising an engineered TnT polypeptide fused to a second polypeptide with inorganic pyrophosphatase (IPP) activity (as previously described in PCT/US2023/076667). For example, synthetic genes encoding an *N*-terminal and *C*-terminal hexahistidine tagged version of a wild-type (WT) inorganic pyrophosphatase (IPP) polypeptide can be fused to gene encoding a TnT variant polypeptide. Typically, the polypeptides (e.g., IPP and TnT) are fused via a polypeptide linker (e.g., a GSGGTG linker) introduced in the construct between the genes encoding the polypeptides. Such fusion proteins can be constructed using well-established techniques (e.g., Gibson assembly cloning) and expressed in *E. coli* (e.g., a strain derived from W3110). It is contemplated that any of the embodiments an engineered TnT polypeptides of even-numbered sequence set forth in SEQ ID NO: 2-1056, 1130-1880, and 1928-3008 could be used in such a fusion with a second polypeptide.

[0279] In some embodiments, the engineered TnT polypeptides of the invention is immobilized. In some embodiments, the TdT or template-independent polymerase is simultaneously purified and immobilized on a solid support. In some embodiments, the immobilized TdT or template-independent polymerase is an engineered TdT with greater than 60% sequence identity to SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008 and one or more substitutions or substitution sets in the amino acid sequence of the engineered TdT. In some embodiments, the immobilized TdT or template-independent polymerase is immobilized on a solid support.

[0280] In some embodiments, the engineered TdT polypeptides can be provided on a solid support, such as a membrane, resin, solid carrier, or other solid phase material. A solid support can be composed of organic polymers such as polystyrene, polyethylene, polypropylene, polyfluoroethylene, polyethyleneoxy, and polyacrylamide, as well as co-polymers and grafts thereof. A solid support can also be inorganic, such as glass, silica, controlled pore glass (CPG), reverse phase silica or metal, such as gold or platinum. The configuration of a solid support can be in the form of beads, spheres, particles, granules, a gel, a membrane, or a surface. Surfaces can be planar, substantially planar, or non-planar. Solid supports can be porous or non-porous and can have swelling or non-swelling characteristics. A solid support can be configured in the form of a well, depression, or other container, vessel, feature, or location.

[0281] In some embodiments, the engineered TdT polypeptides of the present invention can be immobilized on a solid support such that they retain their improved activity, and/or other improved properties relative to the reference polypeptide of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002. In such embodiments, the immobilized polypeptides can facilitate the biocatalytic conversion of the substrate compounds or other suitable substrates to the product and after the reaction is complete are easily retained (e.g., by retaining beads on which polypeptide is immobilized) and then reused or recycled in subsequent reactions.

[0282] Methods of enzyme immobilization are well-known in the art. The engineered polypeptides can be bound non-covalently or covalently. Various methods for conjugation and immobilization of enzymes to solid supports (e.g., resins, membranes, beads, glass, etc.) are well known in the art (See e.g., Yi et al., Proc. Biochem., 2007, 42(5):895-898; Martin et al., Appl. Microbiol. Biotechnol., 2007, 76(4):843-851; Koszelewski et al., J. Mol. Cat. B: Enzymatic, 2010, 63: 39-44; Truppo et al., Org. Proc. Res. Dev., published online: dx.doi.org/10.1021/op200157c; Hermanson, Bioconjugate Techniques, 2nd ed., Academic Press, Cambridge, MA (2008); Mateo et al., Biotechnol. Prog., 2002, 18(3):629-34; and "Bioconjugation Protocols: Strategies and Methods," *In* Methods in Molecular Biology, Niemeyer (ed.), Humana Press, New York, NY ((2004); the disclosures of each which are incorporated by reference herein). Solid supports useful for immobilizing the engineered TdT of the present invention include but are not limited to beads or resins comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups. Exemplary solid supports useful for immobilizing the engineered TdT polypeptides of the present invention include, but are not limited to, EnginZyme (including, EziG-1, EziG-1, and EziG-3), chitosan beads, Eupergit C, and SEPABEADs (Mitsubishi) (including EC-EP, EC-HFA/S, EXA252, EXE119 and EXE120).

[0283] It is to be understood that the polypeptides described herein are not restricted to the genetically encoded amino acids. In addition to the genetically encoded amino acids, the polypeptides described herein may be comprised, either in whole or in part, of naturally occurring and/or synthetic non-encoded amino acids. Certain commonly encountered non-encoded amino acids of which the polypeptides described herein may be comprised include, but are not limited to: the D-stereoisomers of the genetically-encoded amino acids; 2,3-diaminopropionic acid (Dpr); α-aminoisobutyric acid (Aib); ε-aminohexanoic acid (Aha); δ-aminovaleric acid (Ava); N-methylglycine or sarcosine (MeGly or Sar); ornithine (Orn); citrulline (Cit); t-butylalanine (Bua); t-butylglycine (Bug); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); naphthylalanine (Nal); 2-chlorophenylalanine (Ocf); 3-chlorophenylalanine (Mcf); 4-chlorophenylalanine (Pcf); 2-fluorophenylalanine (Off); 3-fluorophenylalanine (Mff); 4-fluorophenylalanine (Pff); 2-bromophenylalanine (Obf); 3-bromophenylalanine (Mbf); 4-bromophenylalanine (Pbf); 2-methylphenylalanine (Omf); 3-methylphenylalanine (Mmf); 4-methylphenylalanine (Pmf); 2-nitrophenylalanine (Onf); 3-nitrophenylalanine (Mnf); 4-nitrophenylalanine (Pnf); 2-cyanophenylalanine (Ocf); 3-cyanophenylalanine (Mcf); 4-cyanophenylalanine (Pcf); 2-trifluoromethylphenylalanine (Otf); 3-trifluoromethylphenylalanine (Mtf); 4-trifluoromethylphenylalanine (Ptf); 4-aminophenylalanine (Paf); 4-iodophenylalanine (Pif); 4-aminomethylphenylalanine (Pamf); 2,4-dichlorophenylalanine (Opef); 3,4-dichlorophenylalanine (Mpcf); 2,4-difluorophenylalanine (Opff); 3,4-difluorophenylalanine (Mpff); pyrid-2-ylalanine (2pAla); pyrid-3-ylalanine (3pAla); pyrid-4-ylalanine (4pAla); naphth-1-ylalanine (1nAla); naphth-2-ylalanine (2nAla); thiazolylalanine (taAla); benzothienylalanine (bAla); thienylalanine (tAla); furylalanine (fAla); homophenylalanine (hPhe); homotyrosine (hTyr); homotryptophan (hTrp); pentafluorophenylalanine (5ff); styrylkalanine (sAla); authrylalanine (aAla); 3,3-diphenylalanine (Dfa); 3-amino-5-phenypentanoic acid (Afp); penicillamine (Pen); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β-2-thienylalanine (Thi); methionine sulfoxide (Mso); N(w)-nitroarginine (nArg); homolysine (hLys); phosphonomethylphenylalanine (pmPhe); phosphoserine (pSer); phosphothreonine (pThr); homoaspartic acid (hAsp); homoglutanic acid

(hGlu); 1-aminocyclopent-(2 or 3)-ene-4 carboxylic acid; pipecolic acid (PA), azetidine-3-carboxylic acid (ACA); 1-aminocyclopentane-3-carboxylic acid; allylglycine (aGly); propargylglycine (pgGly); homoalanine (hAla); norvaline (nVal); homoleucine (hLeu), homovaline (hVal); homoisoleucine (hIle); homoarginine (hArg); N-acetyl lysine (AcLys); 2,4-diaminobutyric acid (Dbu); 2,3-diaminobutyric acid (Dab); N-methylvaline (MeVal); homocysteine (hCys); homoserine (hSer); hydroxyproline (Hyp) and homoproline (hPro). Additional non-encoded amino acids of which the polypeptides described herein may be comprised will be apparent to those of skill in the art (See e.g., the various amino acids provided in Fasman, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Boca Raton, FL, pp. 3-70 (1989), and the references cited therein, all of which are incorporated by reference). These amino acids may be in either the L- or D-configuration.

[0284] Those of skill in the art will recognize that amino acids or residues bearing side chain protecting groups may also comprise the polypeptides described herein. Non-limiting examples of such protected amino acids, which in this case belong to the aromatic category, include (protecting groups listed in parentheses), but are not limited to: Arg(tos), Cys(methylbenzyl), Cys (nitropyridinesulfenyl), Glu(δ-benzylester), Gln(xanthyl), Asn(N-δ-xanthyl), His(bom), His(benzyl), His(tos), Lys(fmoc), Lys(tos), Ser(O-benzyl), Thr (O-benzyl) and Tyr(O-benzyl).

[0285] Non-encoding amino acids that are conformationally constrained of which the polypeptides described herein may be composed include, but are not limited to, N-methyl amino acids (L-configuration); 1-aminocyclopent-(2 or 3)-ene-4-carboxylic acid; pipecolic acid; azetidine-3-carboxylic acid; homoproline (hPro); and 1-aminocyclopentane-3-carboxylic acid.

[0286] In some embodiments, the engineered polypeptides can be in various forms, for example, such as an isolated preparation, as a substantially purified enzyme, whole cells transformed with gene(s) encoding the enzyme, and/or as cell extracts and/or lysates of such cells. The enzymes can be lyophilized, spray-dried, precipitated or be in the form of a crude paste, as further discussed below.

[0287] In some embodiments, the engineered polypeptides can be in the form of a biocatalytic composition. In some embodiments, the biocatalytic composition comprises (a) a means for conversion of an NTP-3-O-RBG or natural or modified NTP substrate and an oligo acceptor compound to an oligo acceptor product extended by one nucleotide by contact with a TnT and (b) a suitable cofactor. The suitable cofactor may be cobalt, manganese, or any other suitable cofactor.

[0288] In some embodiments, the polypeptides described herein are provided in the form of kits. The enzymes in the kits may be present individually or as a plurality of enzymes. The kits can further include reagents for carrying out the enzymatic reactions, substrates for assessing the activity of enzymes, as well as reagents for detecting the products. The kits can also include reagent dispensers and instructions for use of the kits.

[0289] In some embodiments, the kits of the present invention include arrays comprising a plurality of different TnT polypeptides at different addressable position, wherein the different polypeptides are different variants of a reference sequence each having at least one different improved enzyme property. In some

embodiments, a plurality of polypeptides immobilized on solid supports are configured on an array at various locations, addressable for robotic delivery of reagents, or by detection methods and/or instruments. The array can be used to test a variety of substrate compounds for conversion by the polypeptides. Such arrays comprising a plurality of engineered polypeptides and methods of their use are known in the art (See e.g., WO2009/008908A2).

Polynucleotides, Expression Vectors and Host Cells

[0290] In another aspect, the present invention provides polynucleotides encoding the engineered TnT polypeptides described herein. The polynucleotides may be operatively linked to one or more heterologous regulatory sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. Expression constructs containing a heterologous polynucleotide encoding the engineered TnT are introduced into appropriate host cells to express the corresponding TnT polypeptide.

[0291] As will be apparent to the skilled artisan, availability of a protein sequence and the knowledge of the codons corresponding to the various amino acids provide a description of all the polynucleotides capable of encoding the subject polypeptides. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons, allows an extremely large number of nucleic acids to be made, all of which encode the improved TnT enzymes. Thus, having knowledge of a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the protein. In this regard, the present invention specifically contemplates each and every possible variation of polynucleotides that could be made encoding the polypeptides described herein by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide described herein, including the amino acid sequences presented in **Tables 5.2**, **6.2**, **7.2**, **8.2**, **9.2**, **10.2**, **11.2**, **12.2**, **13.2**, **14.2**, **15.2**, **16.2**, **17.2**, **18.2**, **19.2**, **20.2**, **21.2**, **22.2**, **23.2**, **24.2**, **25.2**, **26.2**, **27.2**, **28.2**, **29.2**, **30.2**, **35.2**, **36.2**, **37.2**, **38.2**, **39.2**, **40.2**, **41.2**, **42.2**, **43.2**, **44.2**, **45.2**, **46.2**, **47.2**, **48.2**, **49.2**, **50.2**, **51.2**, **52.2**, **53.2**, **54.2**, **56.2**, **57.2**, **58.2**, **59.2**, **60.2**, **61.2**, **62.2**, **63.2**, **64.2**, **65.2**, **66.2**, **67.2**, **68.2**, **69.2**, **70.2**, **71.2**, **72.2**, **73.2**, **74.2**, **75.2**, **76.2**, **77.2**, **78.2**, **79.2**, **80.2**, **81.2**, **82.2**, **83.2**, **84.2**, **85.2**, **86.2**, **87.2**, and **88.2**, and disclosed in the Sequence Listing incorporated by reference herein as the even-numbered sequences (SEQ ID NO.) in the range of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0292] In various embodiments, the codons are preferably selected to fit the host cell in which the protein is being produced. For example, preferred codons used in bacteria are used to express the gene in bacteria; preferred codons used in yeast are used for expression in yeast; and preferred codons used in mammals are used for expression in mammalian cells. In some embodiments, all codons need not be replaced to optimize the codon usage of the TnT since the natural sequence will comprise preferred codons and because use of preferred codons may not be required for all amino acid residues. Consequently, codon optimized polynucleotides encoding the TnT enzymes may contain preferred codons at about 40%, 50%, 60%, 70%, 80%, or greater than 90% of codon positions of the full-length coding region.

[0293] In some embodiments, the polynucleotide has a nucleic acid sequence comprising at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, 99%, or more identity to the codon optimized nucleic acid sequences encoding an amino acid sequence corresponding to amino acid residues 12 to carboxy terminus of an even-numbered sequence in the range of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008, or an amino acid sequence of an even-numbered sequence in the range of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008.

[0294] In some embodiments, the polynucleotide comprises a codon optimized nucleotide sequence encoding the TnT polypeptide amino acid sequence, as represented by the sequence comprising amino acid residues 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or the sequence comprising SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0295] In some embodiments, the polynucleotide comprises a polynucleotide sequence encoding at least an engineered terminal nucleotidyl transferase comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to the reference sequence corresponding to amino acid residue 12 to carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0296] In some embodiments, the polynucleotide comprises a polynucleotide sequence encoding at least an engineered terminal nucleotidyl transferase comprising an amino acid sequence comprising at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, or 526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742,

1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0297] In some embodiments, the polynucleotide has a nucleic acid sequence comprising at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identity to the codon optimized nucleic acid sequence corresponding to nucleotide residues 34 to 3'-terminus of an odd-numbered sequence in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007, or the codon optimized nucleic acid sequence corresponding to an odd-numbered sequence in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007. In some embodiments, the codon optimized sequence corresponding to nucleotide residues 34 to 3'-terminus of an odd-numbered sequences in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007, or the codon optimized sequence corresponding to an odd-numbered sequences in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007, enhance expression of the encoded TnT, providing preparations of enzyme capable of converting substrate to product.

[0298] In some embodiments, the polynucleotide comprises a polynucleotide sequence comprising at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to the reference nucleotide sequence corresponding to nucleotide residues 32 to 3'-terminus of SEQ ID NOs: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2561, 2713, 2875, 2883, 2909, 2973, 2997, or 3001, or to the reference nucleotide sequence corresponding to SEQ ID NOs: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2561, 2713, 2875, 2883, 2909, 2973, 2997, or 3001, and/or or a functional fragment thereof, wherein the polynucleotide sequence encodes an engineered polypeptide comprising at least one substitution at one or more amino acid positions, as described herein.

[0299] In some embodiments, the polynucleotide sequence comprises nucleotide residues 34 to 3'-terminus of SEQ ID NO: 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2561, 2713, 2875, 2883, 2909, 2973, 2997, or 3001, or comprises SEQ ID NO: 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2561, 2713, 2875, 2883, 2909, 2973, 2997, or 3001.

[0300] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a reference sequence selected from the odd-numbered sequences in SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007 or a complement thereof, and encode a TnT.

[0301] In some embodiments, as described above, the polynucleotide encodes an engineered TnT polypeptide with improved properties as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668,

682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, wherein the polypeptide comprises an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to a reference sequence selected from SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, and one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, wherein the sequence is selected from the even-numbered sequences in the range of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008. In some embodiments, the reference amino acid sequence is selected from the even-numbered sequences in the range of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2, while in some other embodiments, the reference sequence is SEQ ID NO: 8, while in some other embodiments, the reference sequence is SEQ ID NO: 70. In some embodiments, the reference amino acid sequence is SEQ ID NO: 148, while in some other embodiments, the reference sequence is SEQ ID NO: 184, while in some other embodiments, the reference sequence is SEQ ID NO: 234. In some embodiments, the reference amino acid sequence is SEQ ID NO: 256, while in some other embodiments, the reference sequence is SEQ ID NO: 478, while in some other embodiments, the reference sequence is SEQ ID NO: 668. In some embodiments, the reference amino acid sequence is SEQ ID NO: 682, while in some other embodiments, the reference sequence is SEQ ID NO: 730, while in some other embodiments, the reference sequence is SEQ ID NO: 742. In some embodiments, the reference amino acid sequence is SEQ ID NO: 932, while in some other embodiments, the reference sequence is SEQ ID NO: 734. In some embodiments, the reference amino acid sequence is SEQ ID NO: 992, while in some other embodiments, the reference sequence is SEQ ID NO: 1144. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1212, while in some other embodiments, the reference sequence is SEQ ID NO: 1236. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1264, while in some other embodiments, the reference sequence is SEQ ID NO: 1414. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1504, while in some other embodiments, the reference sequence is SEQ ID NO: 1522. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1702, while in some other embodiments, the reference sequence is SEQ ID NO: 1742. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1928, while in some other embodiments, the reference sequence is SEQ ID NO: 1944. In some embodiments, the reference amino acid sequence is SEQ ID NO: 1946, while in some other embodiments, the reference sequence is SEQ ID NO: 1984. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2064, while in some other embodiments, the reference sequence is SEQ ID NO: 2104. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2210, while in some other embodiments, the reference sequence is SEQ ID NO: 2256. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2280, while in some other embodiments, the reference sequence is SEQ ID NO: 2342. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2438, while in some other embodiments, the reference sequence is SEQ ID NO:

2452. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2562, while in some other embodiments, the reference sequence is SEQ ID NO: 2576. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2714, while in some other embodiments, the reference sequence is SEQ ID NO: 2876. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2884, while in some other embodiments, the reference sequence is SEQ ID NO: 2910. In some embodiments, the reference amino acid sequence is SEQ ID NO: 2974, while in some other embodiments, the reference sequence is SEQ ID NO: 2998.

[0302] In some embodiments, the polynucleotide encodes a TnT polypeptide capable of converting one or more substrates to product with improved properties as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, wherein the polypeptide comprises an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to reference sequence of SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0303] In some embodiments, the polynucleotide encoding the engineered TnT comprises a polynucleotide sequence selected from the odd-numbered sequences in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007.

[0304] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a reference polynucleotide sequence selected from the odd-numbered sequences in the range of SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007, or a complement thereof, and encode a TnT polypeptide with one or more of the improved properties described herein. In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes a TnT comprising an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NOs: 2, 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, that has an amino acid sequence comprising one or more residue differences as compared to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, as described above and in the Examples, below.

[0305] In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes an engineered TnT polypeptide with improved properties comprising an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002. In some embodiments, the

polynucleotides encode the polypeptides described herein but have at least about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity at the nucleotide level to a reference polynucleotide encoding the engineered TnT. In some embodiments, the reference polynucleotide sequence is selected from SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007.

[0306] In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes an engineered TnT polypeptide with improved properties comprising an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002. In some embodiments, the polynucleotides encode the polypeptides described herein but have at least about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity at the nucleotide level to a reference polynucleotide encoding the engineered TnT. In some embodiments, the reference polynucleotide sequence is selected from SEQ ID NOs: 1-1055, 1129-1879, and 1927-3007.

[0307] In some embodiments, an isolated polynucleotide encoding any of the engineered TnT polypeptides provided herein is manipulated in a variety of ways to provide for expression of the polypeptide. In some embodiments, the polynucleotides encoding the polypeptides are provided as expression vectors where one or more control sequences is present to regulate the expression of the polynucleotides and/or polypeptides. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art.

[0308] In some embodiments, the control sequences include among other sequences, promoters, leader sequences, polyadenylation sequences, propeptide sequences, signal peptide sequences, and transcription terminators. As known in the art, suitable promoters can be selected based on the host cells used. For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present application, include, but are not limited to the promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (dagA), *Bacillus subtilis* levansucrase gene (sacB), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, and prokaryotic beta-lactamase gene (See e.g., Villa-Kamaroff et al., Proc. Natl Acad. Sci. USA, 1978, 75: 3727-373), as well as the *tac* promoter (See e.g., DeBoer et al., Proc. Natl Acad. Sci. USA, 1983, 80: 21-25). Exemplary promoters for filamentous fungal host cells, include promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (See e.g., WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate

isomerase), and mutant, truncated, and hybrid promoters thereof. Exemplary yeast cell promoters can be from the genes can be from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are known in the art (See e.g., Romanos et al., Yeast. 1992, 8:423-488).

[0309] In some embodiments, the control sequence is a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice finds use in the present invention. For example, exemplary transcription terminators for filamentous fungal host cells can be obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease. Exemplary terminators for yeast host cells can be obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are known in the art (See e.g., Romanos et al., *supra*).

[0310] In some embodiments, the control sequence is a suitable leader sequence, a non-translated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used. Exemplary leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase. Suitable leaders for yeast host cells include but are not limited to those obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP). The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells include but are not limited to those from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are also known in the art (See e.g., Guo and Sherman, Mol. Cell. Bio., 1995, 15:5983-5990).

[0311] In some embodiments, the control sequence is a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. Any signal peptide coding region that

directs the expressed polypeptide into the secretory pathway of a host cell of choice finds use for expression of the engineered TnT polypeptides provided herein. Effective signal peptide coding regions for bacterial host cells include but are not limited to the signal peptide coding regions obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. Further signal peptides are known in the art (See e.g., Simonen and Palva, Microbiol. Rev., 1993, 57:109-137). Effective signal peptide coding regions for filamentous fungal host cells include but are not limited to the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase. Useful signal peptides for yeast host cells include but are not limited to those from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase.

[0312] In some embodiments, the control sequence is a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is referred to as a "proenzyme," "propolypeptide," or "zymogen," in some cases). A propolypeptide can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region includes but is not limited to the genes for *Bacillus subtilis* alkaline protease (aprE), *Bacillus subtilis* neutral protease (nprT), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* lactase (See e.g., WO 95/33836). Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

[0313] In some embodiments, regulatory sequences are also utilized. These sequences facilitate the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include, but are not limited to the *lac*, *tac*, and *trp* operator systems. In yeast host cells, suitable regulatory systems include, but are not limited to the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include, but are not limited to the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter.

[0314] The present invention also provides recombinant expression vectors comprising a polynucleotide encoding an engineered TnT polypeptide, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. In some embodiments, the various nucleic acid and control sequences described above are combined together to produce a recombinant expression vector which includes one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the variant TnT polypeptide at such sites. Alternatively, the polynucleotide sequence(s) of the present invention are expressed by inserting the polynucleotide sequence or a nucleic acid construct comprising the polynucleotide sequence into an

appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0315] The recombinant expression vector may be any vector (e.g., a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and can result in the expression of the variant TnT polynucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[0316] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extra-chromosomal element, a minichromosome, or an artificial chromosome). The vector may contain any means for assuring self-replication. In some alternative embodiments, the vector may be one which, when introduced into the host cell, is integrated into the genome, and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

[0317] In some embodiments, the expression vector preferably contains one or more selectable markers, which permit easy selection of transformed cells. A "selectable marker" is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophy, and the like. Examples of bacterial selectable markers include but are not limited to the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, amdS (acetamidase), argB (ornithine carbamoyltransferases), bar (phosphinothricin acetyltransferase), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate adenyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. In another aspect, the present invention provides a host cell comprising a polynucleotide encoding at least one engineered TnT polypeptide of the present invention, the polynucleotide being operatively linked to one or more control sequences for expression of the engineered TnT enzyme(s) in the host cell. Host cells for use in expressing the polypeptides encoded by the expression vectors of the present invention are well known in the art and include but are not limited to, bacterial cells, such as *E. coli*, *Vibrio fluvialis*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (e.g., *Saccharomyces cerevisiae* and *Pichia pastoris* [ATCC Accession No. 201178]); insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, BHK, 293, and Bowes melanoma cells; and plant cells. Exemplary host cells are *Escherichia coli* strains (e.g., W3110 (ΔfhuA) and BL21).

[0318] In some embodiments, the host cell strain comprises a knockout of one or more genes, in particular phosphatase genes. In some embodiments, the host cell comprises a knockout or single gene deletion of *E. coli genes aphA*, *surE*, *phoA*, and/or *cpdB*, as described below in the Examples. In some embodiments, the host

cell comprising a knockout of one or more phosphatase genes has increased production of the product and/or decreased de-phosphorylation of the product or substrate.

[0319] Accordingly, in another aspect, the present invention provides methods for producing the engineered TnT polypeptides, where the methods comprise culturing a host cell capable of expressing a polynucleotide encoding the engineered TnT polypeptide under conditions suitable for expression of the polypeptide. In some embodiments, the methods further comprise the steps of isolating and/or purifying the TnT polypeptides, as described herein.

[0320] Appropriate culture media and growth conditions for the above-described host cells are well known in the art. Polynucleotides for expression of the TnT polypeptides may be introduced into cells by various methods known in the art. Techniques include, among others, electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion.

[0321] The engineered TnTs with the properties disclosed herein can be obtained by subjecting the polynucleotide encoding the naturally occurring or engineered TnT polypeptide to mutagenesis and/or directed evolution methods known in the art, and as described herein. An exemplary directed evolution technique is mutagenesis and/or DNA shuffling (See e.g., Stemmer, Proc. Natl. Acad. Sci. USA, 1994, 91:10747-10751; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767 and U.S. Pat. 6,537,746). Other directed evolution procedures that can be used include, among others, staggered extension process (StEP), in vitro recombination (See e.g., Zhao et al., Nat. Biotechnol., 1998, 16:258–261), mutagenic PCR (See e.g., Caldwell et al., PCR Methods Appl., 1994, 3:S136-S140), and cassette mutagenesis (See e.g., Black et al., Proc. Natl. Acad. Sci. USA, 1996, 93:3525-3529).

[0322] For example, mutagenesis and directed evolution methods can be readily applied to polynucleotides to generate variant libraries that can be expressed, screened, and assayed. Mutagenesis and directed evolution methods are well known in the art (See e.g., US Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, 5,837,458, 5,928,905, 6,096,548, 6,117,679, 6,132,970, 6,165,793, 6,180,406, 6,251,674, 6,265,201, 6,277,638, 6,287,861, 6,287,862, 6,291,242, 6,297,053, 6,303,344, 6,309,883, 6,319,713, 6,319,714, 6,323,030, 6,326,204, 6,335,160, 6,335,198, 6,344,356, 6,352,859, 6,355,484, 6,358,740, 6,358,742, 6,365,377, 6,365,408, 6,368,861, 6,372,497, 6,337,186, 6,376,246, 6,379,964, 6,387,702, 6,391,552, 6,391,640, 6,395,547, 6,406,855, 6,406,910, 6,413,745, 6,413,774, 6,420,175, 6,423,542, 6,426,224, 6,436,675, 6,444,468, 6,455,253, 6,479,652, 6,482,647, 6,483,011, 6,484,105, 6,489,146, 6,500,617, 6,500,639, 6,506,602, 6,506,603, 6,518,065, 6,519,065, 6,521,453, 6,528,311, 6,537,746, 6,573,098, 6,576,467, 6,579,678, 6,586,182, 6,602,986, 6,605,430, 6,613,514, 6,653,072, 6,686,515, 6,703,240, 6,716,631, 6,825,001, 6,902,922, 6,917,882, 6,946,296, 6,961,664, 6,995,017, 7,024,312, 7,058,515, 7,105,297, 7,148,054, 7,220,566, 7,288,375, 7,384,387, 7,421,347, 7,430,477, 7,462,469, 7,534,564, 7,620,500, 7,620,502, 7,629,170, 7,702,464, 7,747,391, 7,747,393, 7,751,986, 7,776,598, 7,783,428, 7,795,030, 7,853,410, 7,868,138, 7,783,428, 7,873,477, 7,873,499, 7,904,249, 7,957,912, 7,981,614, 8,014,961, 8,029,988, 8,048,674, 8,058,001, 8,076,138, 8,108,150, 8,170,806, 8,224,580, 8,377,681, 8,383,346, 8,457,903, 8,504,498, 8,589,085, 8,762,066, 8,768,871, 9,593,326, and all related US, as well as PCT and non-US counterparts; Ling et al., Anal. Biochem., 1997, 254(2):157-78; Dale et al., Meth. Mol.

Biol., 1996, 57:369-74; Smith, Ann. Rev. Genet., 1985, 19:423-462; Botstein et al., Science, 1985, 229:1193-1201; Carter, Biochem. J., 1986, 237:1-7; Kramer et al., Cell, 1984, 38:879-887; Wells et al., Gene, 1985, 34:315-323; Minshull et al., Curr. Op. Chem. Biol., 1999, 3:284-290; Christians et al., Nat. Biotechnol., 1999, 17:259-264; Crameri et al., 1998, Nature, 391:288-291; Crameri, et al., Nat. Biotechnol., 1997, 15:436-438; Zhang et al., Proc. Nat. Acad. Sci. USA, 1997, 94:4504-4509; Crameri et al., Nat. Biotechnol., 1996, 14:315-319; Stemmer, Nature, 1994, 370:389-391; Stemmer, Proc. Nat. Acad. Sci. USA, 1994, 91:10747-10751; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; and WO 2009/152336, all of which are incorporated herein by reference).

[0323] In some embodiments, the enzyme clones obtained following mutagenesis treatment are screened by subjecting the enzymes to a defined temperature (or other assay conditions, such as testing the enzyme's activity over a broad range of substrates) and measuring the amount of enzyme activity remaining after heat treatments or other assay conditions. Clones containing a polynucleotide encoding a TnT polypeptide are then sequenced to identify the nucleotide sequence changes (if any) and used to express the enzyme in a host cell. Measuring enzyme activity from the expression libraries can be performed using any suitable method known in the art (e.g., standard biochemistry techniques, such as HPLC analysis).

[0324] In some embodiments, the clones obtained following mutagenesis treatment can be screened for engineered TnTs having one or more desired improved enzyme properties (e.g., improved regioselectivity). Measuring enzyme activity from the expression libraries can be performed using the standard biochemistry techniques, such as HPLC analysis, LC-MS analysis, RapidFire-MS analysis, and/or capillary electrophoresis analysis.

[0325] When the sequence of the engineered polypeptide is known, the polynucleotides encoding the enzyme can be prepared by standard solid-phase methods, according to known synthetic methods. In some embodiments, fragments of up to about 100 bases can be individually synthesized, then joined (*e.g.*, by enzymatic or chemical ligation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides encoding portions of the TnT can be prepared by chemical synthesis as known in the art (e.g., the classical phosphoramidite method of Beaucage et al., Tet. Lett., 1981, 22:1859-69, or the method described by Matthes et al., EMBO J., 1984, 3:801-05) as typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized (*e.g.*, in an automatic DNA synthesizer), purified, annealed, ligated, and cloned in appropriate vectors. In addition, essentially any nucleic acid can be obtained from any of a variety of commercial sources. In some embodiments, additional variations can be created by synthesizing oligonucleotides containing deletions, insertions, and/or substitutions, and combining the oligonucleotides in various permutations to create engineered TnTs with improved properties.

[0326] Accordingly, in some embodiments, a method for preparing the engineered TnT polypeptide comprises: (a) synthesizing a polynucleotide encoding a polypeptide comprising an amino acid sequence having at least about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to an amino acid sequence selected from the even-numbered sequences of SEQ ID NOs: 2-1056, 1130-1880, and 1928-3008, and having one or more residue differences as compared to

SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002; and (b) expressing the TnT polypeptide encoded by the polynucleotide.

[0327] In some embodiments of the method, the polynucleotide encodes an engineered TnT that has optionally one or several (e.g., up to 3, 4, 5, or up to 10) amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-75, 1-100, or 1-150 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally around 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the substitutions can be conservative or non-conservative substitutions.

[0328] In some embodiments, any of the engineered TnT enzymes expressed in a host cell can be recovered from the cells and/or the culture medium using any one or more of the well-known techniques for protein purification, including, among others, lysozyme treatment, sonication, filtration, salting-out, ultra-centrifugation, and chromatography. Suitable solutions for lysing and the high efficiency extraction of proteins from bacteria, such as *E. coli*, are commercially available (e.g., CelLytic B™, Sigma-Aldrich, St. Louis MO).

[0329] Chromatographic techniques for isolation of the TnT polypeptide include, among others, reverse phase chromatography high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., and will be apparent to those having skill in the art.

[0330] In some embodiments, affinity techniques may be used to isolate the improved TnT enzymes. For affinity chromatography purification, any antibody which specifically binds the TnT polypeptide may be used. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with a TnT polypeptide, or a fragment thereof. The TnT polypeptide or fragment may be attached to a suitable carrier, such as BSA, by means of a side chain functional group or linkers attached to a side chain functional group. In some embodiments, the affinity purification can use a specific ligand bound by the TnT or dye affinity column (See e.g., EP0641862; Stellwagen, "Dye Affinity Chromatography," *In* Current Protocols in Protein Science, Unit 9.2-9.2.16 (2001)).

Methods of Using the Engineered TnT Enzymes

[0331] In some embodiments, the TnT enzymes described herein find use in processes for conversion of one or more suitable substrates to a product.

[0332] In some embodiments, the engineered TnT polypeptides disclosed herein can be used in a process for the conversion of the oligo acceptor substrate and an NTP-3'-O-RBG or natural or modified NTP substrate to a product comprising an oligo acceptor substrate extended by one nucleotide.

[0333] In the embodiments provided herein and illustrated in the Examples, various ranges of suitable reaction conditions that can be used in the processes, include but are not limited to, substrate loading, co-substrate loading, pH, temperature, buffer, solvent system, cofactor, polypeptide loading, and reaction time. Further suitable reaction conditions for carrying out the process for biocatalytic conversion of substrate compounds to product compounds using an engineered TnT described herein can be readily optimized in view of the guidance provided herein by routine experimentation that includes, but is not limited to, contacting the engineered TnT polypeptide and one or more substrate compounds under experimental reaction conditions of concentration, pH, temperature, and solvent conditions, and detecting the product compound.

[0334] The oligo acceptor substrate may be any nucleotide chain or similar moiety with an exposed 3'-OH. In some embodiments, the acceptor substrate may be single stranded. In yet other embodiments, the acceptor substrate may be double stranded or partially doubled stranded. In some embodiments, the acceptor substrate may comprise a nucleotide chain consisting of 1-10 nucleotides, 5-20 nucleotides, 15-50 nucleotides, 30-100 nucleotides, or greater than 100 nucleotides. In some embodiments, the oligo acceptor substrate may comprise a chemical moiety that is not a nucleotide chain but contains a free -OH capable of being recognized as a substrate by a wild-type or engineered TnT.

[0335] In some embodiments, the oligo acceptor substrate may comprise a nucleotide chain consisting of three to seven nucleotides. In some embodiments, the oligo acceptor oligo acceptor substrate comprising three to seven nucleotides may comprise additional modifications, as described herein. In some specific embodiments, the oligo acceptor substrate may comprise a nucleotide chain consisting of three to seven nucleotides and a phosphate at the 5' end of the oligo acceptor substrate.

[0336] In some embodiments, the oligo acceptor substrate may comprise one or more nucleotides with a 2' modification, as described herein. In some embodiments, the oligo acceptor substrate may comprise one or more nucleotides with a 2' modification selected from 2'-OH, 2'-H, 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R' (where R' is any alkyl or aryl), or another 2' atom or chemical group. In some embodiments, the oligo acceptor substrate may comprise one or more additional modifications, such as a phosphorothioate linkage.

[0337] In some embodiments, the sugar may have other modifications at other positions, such as locked nucleotides or constrained ethyl nucleotides, as is known in the art. In some embodiments, "locked nucleoside" or "locked nucleotide" or "locked nucleic acid" (LNA) refers to a nucleoside, nucleotide or nucleic acid, respectively, in which the ribose moiety is modified with a bridge connecting the 2' oxygen and 4' carbon. The LNA may be either in the C3'-endo (beta-D-LNA) or C2'-endo (alpha-L-LNA) conformation (see, e.g., Obika et al., Tetrahedron Letters, 1997, 38(50):8735–8738; Orum et al., Current Pharmaceutical Design, 2008, 14(11):1138–1142). Typically, the bridge is a methylene bridge. LNA may confer additional stability to a polynucleotide.

[0338] In some embodiments, the nucleoside or nucleotide or NTP-RBG may comprise a glycol nucleic acid (GNA) modification, wherein the sugar moiety of the NTP is replaced by propylene glycol. GNA may confer additional stability to a polynucleotide.

[0339] In some embodiments, the 3'-phosphate group of the NQP may act as a removable blocking group or protecting group that may be selectively unblocked or removed to allow further modifications, reactions, or incorporation of the NQP into a growing oligonucleotide chain during template-dependent or template-independent oligonucleotide synthesis.

[0340] In some embodiments, the oligo acceptor substrate comprises a nucleotide chain of repeating nucleotides. In other embodiments, the oligo acceptor substrate comprises a nucleotide chain of varied nucleotides that do not repeat. In some embodiments, the oligo acceptor substrate comprises a nucleotide chain with an odd number of nucleotides. In some embodiments, the oligo acceptor substrate comprises a nucleotide with an even number of nucleotides.

[0341] In some embodiments, the oligo acceptor substate is secured to solid support. Suitable solid supports are known to those in the art and described, below, in this disclosure.

[0342] In some embodiments, the oligo acceptor substrate comprises one or more nucleotide sequences selected from the following 5'-6-FAM-T10mCmCmCmUfA, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T36fCmAmUmC, 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T41*fC*mAfA, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56mA*fC*mAfA, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T51fGmUfGmU, 5'-6-FAM-T11AmC*mA*mG, 5'-6-FAM-T15mAmUmCmU, 5'-6-FAM-T46mUmCmUmU, 5'-6-FAM-T41mAmUmCmU, T8mUfGmUfC, T8fCmAmUmC, T8mCfCmCfG, T8mCfCmGfG, mAmAmAmAmUmCmU, mAmAmAmUmCmU, mAmAmUmCmU, mAmUmCmU, mUmCmU, 5'P-mUmCmU, mC*mA*mGmA, mAfGmUfG, mGmUmC*mC, 5'-6-FAM-T9mAmAmAmUmCmU, 5'-6-FAM-T9mAmAmAmUmCmU(sgna)A, 5'-6-FAM-T9mAmAmAmUmCmU+A, 5'-6-FAM-T9mAmAmAmUmCmU+G, 5'-6-FAM-T9mAmAmAmUmCmU+C, 5'-6-FAM-T9mAmAmAmUmCmU+U, 5'-6-FAM-T9mAmAmAmUmCmU+A+A, 5'-6-FAM-T9mAmAmAmUmCmU+G+G, 5'-6-FAM-T9mAmAmAmUmCmU+U+U, 5'-6-FAM-T16mAfGmAfG, 5'-6-FAM-T26fCfAfUmG, 5'-6-FAM-T10AmC*mA*mG, 5'-6-FAM-T16*fC*mAfAfA, 5'-6-FAM-T16*fA*mAmGfAfG, 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T21mUmAmG*mA, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T25mAmGfUfGfG, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T31mUfCmAmU, 5'-6-FAM-T35fAfGfUmGfG, 5'-6-FAM-T51mAmUmCfC, 5'-6-FAM-T56fGmUfCmU, T8AmC*mA*mG, T8mCfCmGfG, T8*fC*mAfAfA, T8*fA*mAmGfAfG, T8mGfUfAfC, T8fUfUfUfC, T8mAfUfCfC, T8mAmUfUmGfG, T8mAmGfUfGfG, T8mAmGfAmGfG, T8*fU*fUfAfU, T8mGfUmGfG, T8fAmUmUfG, T8mU*fA*fAfG, T8fAfGfUmGfG, T8fGmUfCmU, T8mA*fC*mAfA, as further described in the Examples (e.g., **Tables 5.1**, **6.1**, **7.1**, **8.1**, **9.1**, **10.1**, **11.1**, **12.1**, **13.1**, **14.1**, **15.1**, **16.1**, **17.1**, **18.1**, **19.1**, **20.1**, **21.1**, **22.1**, **23.1**, **24.1**, **25.1**, **26.1**, **27.1**, **28.1**, **29.1**, **30.1**, **31.1**, **32.1**, **35.1**, **36.1**, **37.1**, **38.1**, **39.1**, **40.1**, **41.1**, **42.1**, **43.1**, **44.1**, **45.1**, **46.1**, **47.1**, **48.1**, **49.1**, **50.1**, **51.1**, **52.1**, **53.1**, **54.1**, **56.1**, **57.1**, **58.1**, **59.1**, **60.1**, **61.1**, **62.1**, **63.1**, **64.1**, **65.1**, **66.1**, **67.1**, **68.1**, **69.1**, **70.1**, **71.1**, **72.1**, **73.1**, **74.1**, **75.1**, **76.1**, **77.1**, **78.1**, **79.1**, **80.1**, **81.1**, **82.1**, **83.1**, **84.1**, **85.1**, **86.1**, **87.1**, **88.1**, **89.1**, and **90.1**,, and

the **Oligonucleotide Table**) and the accompanying Sequence Listing. These embodiments are intended to be non-limiting. Any suitable oligo acceptor substrate finds use in the present invention.

[0343] In some embodiments, the NTP-3'-O-RBG substrate comprises a deoxyribonucleoside triphosphate with a 3'-O-RBG. In other embodiments, the NTP-3'-O-RBG substrate may comprise a ribonucleoside triphosphate with a 3'-O-RBG. In yet other embodiments, the NTP-3'-O-RBG substrate may comprise a synthetic nucleoside triphosphate with a 3'-O-RBG. In some embodiments, the NTP-3'-O-RBG substrate may comprise a sugar ring with a number of carbons that is not five. A non-limiting example of this is a threose nucleoside triphosphate.

[0344] A range of 3' removable blocking groups for the NTP-3'-O-RBG substrate useful in the present disclosure are known in the art and include but are not limited to, -O-NH_2, -O-NO_2, -O-PO_3. In some embodiments, the NTP-3'-O-RBG substrate with 3' removable blocking group can be selected from the group consisting of NTP-3'-O-NH_2, NTP-3'-O-NO_2, or NTP-3'-O-PO_3. In some embodiments, the NTP-3'-O-RBG substrate comprises another blocking group that would sterically hinder addition of a second NTP-3'-O-RBG substrate to the 3' end of the growing oligo acceptor substrate strand prior to removal of the removable blocking from the first round of addition.

[0345] In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a natural purine or pyrimidine base, such as adenine, guanine, cytosine, thymine, or uridine. In some embodiments, deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises an unnatural base analog such as inosine, xanthine, hypoxanthine, or another base analog, as is known in the art. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a base with modifications, as is known in the art. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a 2' modification or substitution. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises substitution of an oxygen for a sulfur atom for the creation of phosphorothioate linkages. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises substitution of two oxygens for sulfurs for the creation of phosphorodithioate linkages.

[0346] In some embodiments, the NTP-3'-O-RBG substrate comprises one or more of the following,

2'-methoxyadenosine, 2'-methoxycytidine, 2'-methoxyguanosine, 2'-methoxyuridine, 2'-fluoroadenosine, 2'-fluorocytidine, 2'-fluoroguanosine, 2'-fluorouridine, adenosine ribonucleotide, cytidine ribonucleotide, guanosine ribonucleotide, uridine ribonucleotide, phosphorothioate linkage, 5'-phosphorylation modification, adenosine (S)-glycol nucleic acid, cytidine (S)-glycol nucleic acid, guanosine (S)-glycol nucleic acid, thymidine (S)-glycol nucleic acid, uridine (S)-glycol nucleic acid, C2'-endo locked adenosine ribonucleotide, C2'-endo locked cytidine ribonucleotide, C2'-endo locked guanosine ribonucleotide, C2'-endo locked thymidine ribonucleotide, C2'-endo locked uridine ribonucleotide, 2'-O-(2-methoxyethyl)adenosine, 2'-O-(2-methoxyethyl)cytidine, 2'-O-(2-methoxyethyl)guanosine, 2'-O-(2-methoxyethyl)uridine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxythymidine, 2',3'-dideoxyuridine

and vinylphosphonate 2'O-methoxy uridine, 1-((2S))-1-(2,3-dihydroxypropyl))thymine, 2'O-hexadecyl cytidine.

[0347] The substrate compound(s) in the reaction mixtures can be varied, taking into consideration, for example, the desired amount of product compound, the effect of each substrate concentration on enzyme activity, stability of enzyme under reaction conditions, and the percent conversion of each substrate to product. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.1 μM to 1 μM, 1 μM to 2 μM, 2 μM to 3 μM, 3 μM to 5 μM, 5 μM to 10 μM, or 10 μM to 50 μM., or 50 μM to 100 μM, or 100 μM to 500 μM, or 500 μM to 1000 μM, or 1000 μM to 2000 μM, or 2000 μM to 5000 μM, or 5000 μM to 10000, or 10000 μM or greater. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.5 to about 25 g/L, 1 to about 25 g/L, 5 to about 25 g/L, about 10 to about 25 g/L, or 20 to about 25 g/L. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.5 g/L, at least about 1 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, or at least about 30 g/L, or even greater.

[0348] In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each NTP-3'-O-RBG or natural or modified NTP substrate of at least about 1 μM to 5 μM, 5 μM to 10 μM, 10 μM to 25 μM, 25 μM to 50 μM, 50 μM to 100 μM, 100 μM to 200 μM, 200 μM to 300 μM, 300 μM to 500 μM, 400 μM to 600 μM, 700 μM to 900 μM, 800 μM to 1000 μM, 800 μM to 1200 μM, or 1000 μM to 1500 μM, or 1600 μM to 2500 μM, or 2500 μM to 5000 μM, or 6000 μM to 12000 μM . In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each NTP-3'-O-RBG or natural or modified NTP substrate of at least about 0.5 g/L, at least about 1 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, or at least about 30 g/L, or even greater.

[0349] In carrying out the TnT-mediated synthesis processes described herein, the engineered polypeptide may be added to the reaction mixture in the form of a purified enzyme, partially purified enzyme, whole cells transformed with gene(s) encoding the enzyme, as cell extracts and/or lysates of such cells, and/or as an enzyme immobilized on a solid support. Whole cells transformed with gene(s) encoding the engineered TnT enzyme or cell extracts, lysates thereof, and isolated enzymes may be employed in a variety of different forms, including solid (e.g., lyophilized, spray-dried, and the like) or semisolid (e.g., a crude paste). The cell extracts or cell lysates may be partially purified by precipitation (ammonium sulfate, polyethyleneimine, heat treatment or the like, followed by a desalting procedure prior to lyophilization (e.g., ultrafiltration, dialysis, etc.). Any of the enzyme preparations (including whole cell preparations) may be stabilized by crosslinking using known crosslinking agents, such as, for example, glutaraldehyde or immobilization to a solid phase (e.g., Eupergit C, and the like).

[0350] The gene(s) encoding the engineered TnT polypeptides can be transformed into host cell separately or together into the same host cell. For example, in some embodiments one set of host cells can be transformed with gene(s) encoding one engineered TnT polypeptide, and another set can be transformed with gene(s) encoding another TnT. Both sets of transformed cells can be utilized together in the reaction mixture in the form of whole cells, or in the form of lysates or extracts derived therefrom. In other embodiments, a host cell

can be transformed with gene(s) encoding multiple engineered TnT polypeptides. In some embodiments the engineered polypeptides can be expressed in the form of secreted polypeptides, and the culture medium containing the secreted polypeptides can be used for the TnT reaction.

[0351] In some embodiments, the improved activity of the engineered TnT polypeptides disclosed herein provides for processes wherein higher percentage conversion can be achieved with lower concentrations of the engineered polypeptide. In some embodiments of the process, the suitable reaction conditions comprise an engineered polypeptide amount of about 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 20% (w/w), 30% (w/w), 40% (w/w), 50% (w/w), 75% (w/w), 100% (w/w) or more of substrate compound loading.

[0352] In some embodiments, the engineered polypeptide is present at a molar ratio of engineered polypeptide to substrate of about 50 to 1, 25 to 1, 10 to 1, 5 to 1, 1 to 1, 1 to 5, 1 to 10, 1 to 25 or 1 to 50, 1 to 100, 1 to 500, 1 to 1000, or 1 to 2000. In some embodiments, the engineered polypeptide is present at a molar ratio of engineered polypeptide to substrate from a range of about 50 to 1 to a range of about 1 to 2000.

[0353] In some embodiments, the engineered polypeptide is present at about 0.01 g/L to about 50 g/L; about 0.01 to about 0.1 g/L; about 0.05 g/L to about 50 g/L; about 0.1 g/L to about 40 g/L; about 1 g/L to about 40 g/L; about 2 g/L to about 40 g/L; about 5 g/L to about 40 g/L; about 5 g/L to about 30 g/L; about 0.1 g/L to about 10 g/L; about 0.5 g/L to about 10 g/L; about 1 g/L to about 10 g/L; about 0.1 g/L to about 5 g/L; about 0.5 g/L to about 5 g/L; or about 0.1 g/L to about 2 g/L. In some embodiments, the TnT polypeptide is present at about 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L, 1, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, or 50 g/L.

[0354] In some embodiments, the suitable reaction conditions comprise a divalent metal cofactor. In some embodiments, the divalent metal cofactor is cobalt. In some embodiments, the cobalt (II) chloride is present at concentrations of about 1 to 1200 μM; about 50 to 400 μM; about 100 to 300 μM; or about 200 to 600 μM; about 500 to 1000 μM. In some embodiments, the cobalt (II) chloride is present at concentrations of about 150 μM; about 200 μM; about 250 μM, about 500 μM; about 1000 μM; or about 1200 μM.

[0355] In some embodiments of the reaction, a phosphatase is used to degrade inorganic phosphate and shift the reaction equilibrium toward the oligo acceptor extension product. In some embodiments, the phosphatase is an *E. coli* pyrophosphatase. In some embodiments, the phosphatase is present at a concentration of about 0.0001 to 0.01 units/uL; about 0.001 to 0.005 units/uL; or about 0.002 to 0.003 units/uL. In some embodiments, the phosphatase is present at a concentration of about 0.001 units/uL; about 0.002 units/uL; or about 0.003 units/uL. In some embodiments, the phosphatase is from *Geobacillus zalihae*, *Geobacillus lituanicus*, *Methanococcus aeolicus*, or *Methanotorris igneus*. In some embodiments, the phosphatase is present at a concentration of about 0.01 to 10 μM; about 0.01 to 0.1 μM; or about 0.1 to 1 μM; or about 0.1 to 10 μM. In some embodiments, the phosphatase is present at a concentration of about 0.05 μM; about 0.5 μM; or about 1 μM; or about 2 μM; or about 5 μM; or about 10 μM.

[0356] During the course of the reaction, the pH of the reaction mixture may change. The pH of the reaction mixture may be maintained at a desired pH or within a desired pH range. This may be done by the addition of an acid or a base, before and/or during the course of the reaction. Alternatively, the pH may be controlled by

using a buffer. Accordingly, in some embodiments, the reaction condition comprises a buffer. Suitable buffers to maintain desired pH ranges are known in the art and include, by way of example and not limitation, borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), and the like. In some embodiments, the reaction conditions comprise water as a suitable solvent with no buffer present.

[0357] In the embodiments of the process, the reaction conditions comprise a suitable pH. The desired pH or desired pH range can be maintained by use of an acid or base, an appropriate buffer, or a combination of buffering and acid or base addition. The pH of the reaction mixture can be controlled before and/or during the course of the reaction. In some embodiments, the suitable reaction conditions comprise a solution pH from about 4 to about 10, pH from about 5 to about 10, pH from about 5 to about 9, pH from about 6 to about 9, pH from about 6 to about 8. In some embodiments, the reaction conditions comprise a solution pH of about 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 7.8, 8, 8.5, 9, 9.5, or 10.

[0358] In the embodiments of the processes herein, a suitable temperature is used for the reaction conditions, for example, taking into consideration the increase in reaction rate at higher temperatures, and the activity of the enzyme during the reaction time period. Accordingly, in some embodiments, the suitable reaction conditions comprise a temperature of about 10 °C to about 95 °C, about 10 °C to about 75 °C, about 15 °C to about 95 °C, about 20 °C to about 95 °C, about 20 °C to about 65 °C, about 25 °C to about 70 °C, or about 50 °C to about 70 °C. In some embodiments, the suitable reaction conditions comprise a temperature of about 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C or 95 °C. In some embodiments, the temperature during the enzymatic reaction can be maintained at a specific temperature throughout the course of the reaction. In some embodiments, the temperature during the enzymatic reaction can be adjusted over a temperature profile during the course of the reaction.

[0359] In some embodiments, the processes of the invention are carried out in a solvent. Suitable solvents include water, aqueous buffer solutions, organic solvents, polymeric solvents, and/or co-solvent systems, which generally comprise aqueous solvents, organic solvents and/or polymeric solvents. The aqueous solvent (water or aqueous co-solvent system) may be pH-buffered or unbuffered. In some embodiments, the processes using the engineered TnT polypeptides can be carried out in an aqueous co-solvent system comprising an organic solvent (e.g., ethanol, isopropanol (IPA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) ethyl acetate, butyl acetate, 1-octanol, heptane, octane, methyl t butyl ether (MTBE), toluene, and the like), ionic or polar solvents (e.g., 1-ethyl 4 methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl 3 methylimidazolium hexafluorophosphate, glycerol, polyethylene glycols, and the like). In some embodiments, the co-solvent can be a polar solvent, such as a polyol, dimethylsulfoxide (DMSO), or lower alcohol. The non-aqueous co- solvent component of an aqueous co-solvent system may be miscible with the aqueous component, providing a single liquid phase, or may be partly miscible or immiscible with the aqueous component, providing two liquid phases. Exemplary aqueous co-solvent systems can comprise water and one or more co-solvents selected from an organic solvent, polar solvent, and polyol solvent. In general, the co-solvent component of an aqueous co-solvent system is chosen such that it does not adversely inactivate the TnT enzyme under the reaction conditions. Appropriate co-solvent systems can be

readily identified by measuring the enzymatic activity of the specified engineered TnT enzyme with a defined substrate of interest in the candidate solvent system, utilizing an enzyme activity assay, such as those described herein.

[0360] In some embodiments of the process, the suitable reaction conditions comprise an aqueous co-solvent, where the co-solvent comprises DMSO at about 1% to about 50% (v/v), about 1 to about 40% (v/v), about 2% to about 40% (v/v), about 5% to about 30% (v/v), about 10% to about 30% (v/v), or about 10% to about 20% (v/v). In some embodiments of the process, the suitable reaction conditions can comprise an aqueous co-solvent comprising ethanol at about 1% (v/v), about 5% (v/v), about 10% (v/v), about 15% (v/v), about 20% (v/v), about 25% (v/v), about 30% (v/v), about 35% (v/v), about 40% (v/v), about 45% (v/v), or about 50% (v/v).

[0361] In some embodiments, the reaction conditions comprise a surfactant for stabilizing or enhancing the reaction. Surfactants can comprise non-ionic, cationic, anionic and/or amphiphilic surfactants. Exemplary surfactants, include by way of example and not limitation, nonyl phenoxypolyethoxylethanol (NP40), TRITON™ X-100 polyethylene glycol *tert*-octylphenyl ether, polyoxyethylene-stearylamine, cetyltrimethylammonium bromide, sodium oleylamidosulfate, polyoxyethylene-sorbitanmonostearate, hexadecyldimethylamine, etc. Any surfactant that may stabilize or enhance the reaction may be employed. The concentration of the surfactant to be employed in the reaction may be generally from 0.1 to 50 mg/mL, particularly from 1 to 20 mg/mL.

[0362] In some embodiments, the reaction conditions include an antifoam agent, which aids in reducing or preventing formation of foam in the reaction solution, such as when the reaction solutions are mixed or sparged. Anti-foam agents include non-polar oils (e.g., minerals, silicones, etc.), polar oils (e.g., fatty acids, alkyl amines, alkyl amides, alkyl sulfates, etc.), and hydrophobic (e.g., treated silica, polypropylene, etc.), some of which also function as surfactants. Exemplary anti-foam agents include Y-30® (Dow Corning), poly-glycol copolymers, oxy/ethoxylated alcohols, and polydimethylsiloxanes. In some embodiments, the anti-foam can be present at about 0.001% (v/v) to about 5% (v/v), about 0.01% (v/v) to about 5% (v/v), about 0.1% (v/v) to about 5% (v/v), or about 0.1% (v/v) to about 2% (v/v). In some embodiments, the anti-foam agent can be present at about 0.001% (v/v), about 0.01% (v/v), about 0.1% (v/v), about 0.5% (v/v), about 1% (v/v), about 2% (v/v), about 3% (v/v), about 4% (v/v), or about 5% (v/v) or more as desirable to promote the reaction.

[0363] The quantities of reactants used in the TnT reaction will generally vary depending on the quantities of product desired, and concomitantly the amount of substrates employed. Those having ordinary skill in the art will readily understand how to vary these quantities to tailor them to the desired level of productivity and scale of production.

[0364] In some embodiments, the order of addition of reactants is not critical. The reactants may be added together at the same time to a solvent (e.g., monophasic solvent, biphasic aqueous co-solvent system, and the like), or alternatively, some of the reactants may be added separately, and some together at different time points. For example, the cofactor, co-substrate, and substrate may be added first to the solvent.

[0365] The solid reactants (e.g., enzyme, salts, etc.) may be provided to the reaction in a variety of different forms, including powder (e.g., lyophilized, spray dried, and the like), solution, emulsion, suspension, and the like. The reactants can be readily lyophilized or spray dried using methods and equipment that are known to those having ordinary skill in the art. For example, the protein solution can be frozen at -80 °C in small aliquots, then added to a pre-chilled lyophilization chamber, followed by the application of a vacuum.

[0366] For improved mixing efficiency when an aqueous co-solvent system is used, the TnT, and co-substrate may be added and mixed into the aqueous phase first. The substrate may be added and mixed in, followed by the organic phase or the substrate may be dissolved in the organic phase and mixed in. Alternatively, the substrate may be premixed in the organic phase, prior to addition to the aqueous phase.

[0367] The processes of the present invention are generally allowed to proceed until further conversion of substrate to product does not change significantly with reaction time (e.g., less than 10% of substrate being converted, or less than 5% of substrate being converted). In some embodiments, the reaction is allowed to proceed until there is complete or near complete conversion of substrate to product. Transformation of substrate to product can be monitored using known methods by detecting substrate and/or product, with or without derivatization. Suitable analytical methods include gas chromatography, HPLC, MS, and the like. In some embodiments, after suitable conversion to product, the reactants are separated from the oligo acceptor substrate extension product and additional reactants are added to the oligo acceptor substrate extension product to further extend the growing polynucleotide chain. The processes of the present invention may be used to iteratively extend the oligo acceptor extension product until a polynucleotide of a defined sequence and length is synthesized.

[0368] Any of the processes disclosed herein using the engineered polypeptides for the preparation of products can be carried out under a range of suitable reaction conditions, including but not limited to ranges of substrates, temperature, pH, solvent system, substrate loading, polypeptide loading, cofactor loading, and reaction time. In one example, the suitable reaction conditions comprise: (a) oligo acceptor substrate loading of about 50 – 10000 μM of substrate compound; (b) NTP-3'-O-RBG substrate or NTP loading of about 1 – 12000 μM of substrate compound; (c) of about 0.01 g/L to 5 g/L engineered polypeptide; (d) 100 to 5000 μM cobalt (II) chloride; (e) 5 to 100 mM triethanolamine buffer; (f) 0.05 to 10 μM pyrophosphatase; (g) pH at 5-9; and (h) temperature of about 15 °C to 70 °C. In some embodiments, the suitable reaction conditions comprise: (a) oligo acceptor substrate loading of about 2500 μM of substrate compound; (b) NTP-3'-O-RBG or NTP substrate loading of about 3000 μM of substrate compound; (c) of about 0.3 g/L engineered polypeptide; (d) 1000 μM cobalt (II) chloride; (e) 100 mM triethanolamine buffer; (f) 1 μM pyrophosphatase; (g) pH at 7.8; and (h) temperature of about 50 °C. In some embodiments, the enzyme loading is between 1-30% w/w. In some embodiments, additional reaction components or additional techniques carried out to supplement the reaction conditions. These can include taking measures to stabilize or prevent inactivation of the enzyme, reduce product inhibition, shift reaction equilibrium to formation of the desired product.

[0369] In some embodiments, the present disclosure provides an engineered TnT, wherein said engineered TnT has improved activity on NTP-3'-RBGs or modified NTPs, such that NTP-3'-RBGs are incorporated with equivalent efficiency to native NTPs, as compared to another wild-type or engineered TnT. In some

embodiments, the engineered TnT with improved activity on dNTP-3'-O-PO_3, such that dNTP-3'-O-PO_3 is incorporated with equivalent efficiency to native dNTPs, is an engineered TnT polypeptide comprising an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NOs: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

[0370] In further embodiments, any of the above-described processes for the conversion of one or more substrate compounds to product compound can further comprise one or more steps selected from: extraction; isolation; purification; and crystallization of product compound. As is known to those skilled in the art, acidic compounds such as oligonucleotides, NTPs, modified NTPs, and NTP-3'-O-RBGs may exist in various salt forms that can be used interchangeably in the methods described herein. All such forms are specifically envisaged for use in the methods described herein. Methods, techniques, and protocols for extracting, isolating, purifying, and/or crystallizing the product from biocatalytic reaction mixtures produced by the above disclosed processes are known to the ordinary artisan and/or accessed through routine experimentation. Additionally, illustrative methods are provided in the Examples below.

[0371] Various features and embodiments of the invention are illustrated in the following representative examples, which are intended to be illustrative, and not limiting.

EXAMPLES

[0372] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention. Indeed, there are various suitable sources for many of the reagents and equipment described below. It is not intended that the present invention be limited to any particular source for any reagent or equipment item.

[0373] In the experimental disclosure below, the following abbreviations apply: M (molar); mM (millimolar), μM and μM (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); ug and μg (micrograms); L and l (liter); ml and mL (milliliter); ul, uL, μl, and μL (microliter); cm (centimeters); mm (millimeters); μM and μιη (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); psi and PSI (pounds per square inch); °C (degrees Celsius); RT and rt (room temperature); CV (coefficient of variability); CAM and cam (chloramphenicol); LB (lysogeny broth); TB (terrific broth); SFP (shake flask powder); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); nt (nucleotide; polynucleotide); aa (amino acid; polypeptide); E. coli W3110 (commonly used laboratory E. coli strain, available from the Coli Genetic Stock Center [CGSC], New Haven, CT); HTP (high throughput); HPLC (high pressure liquid chromatography); HPLC-UV (HPLC-Ultraviolet Visible Detector); 1H NMR (proton nuclear magnetic resonance spectroscopy); FIOPC (fold improvements over positive control); Sigma and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO; Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Microfluidics (Microfluidics, Westwood, MA); Life Technologies (Life Technologies, a part of Fisher Scientific, Waltham, MA); Amresco (Amresco, LLC, Solon, OH); Carbosynth (Carbosynth, Ltd., Berkshire,

UK); Varian (Varian Medical Systems, Palo Alto, CA); Agilent (Agilent Technologies, Inc., Santa Clara, CA); Infors (Infors USA Inc., Annapolis Junction, MD); and Thermotron (Thermotron, Inc., Holland, MI).

[0374] Abbreviations for modified nucleotides

Abbreviations for modified nucleotides	
alias	**modification**
5'-6-FAM or /56-FAM/	5'-6-carboxyfluorescein modification
T{n}	poly-thymidine of length n
3'P, 3'-P, /3Phos/	3'-phosphorylation modification
mA	2'-methoxyadenosine
mC	2'-methoxycytidine
mG	2'-methoxyguanosine
mU	2'-methoxyuridine
fA, /52FA/, /i2FA/, /32FA/	2'-fluoroadenosine
fC, /52FC/, /i2FC/, /32FC/	2'-fluorocytidine
fG, /52FG/, /i2FG/, /32FG/	2'-fluoroguanosine
fU, /52FU/, /i2FU/, /32FU/	2'-fluorouridine
rA	adenosine ribonucleotide
rC	cytidine ribonucleotide
rG	guanosine ribonucleotide
rU	uridine ribonucleotide
*	phosphorothioate linkage
5'P, 5'-P, /5Phos/	5'-phosphorylation modification
/sgna-A/	adenosine (S)-glycol nucleic acid
/sgna-C/	cytidine (S)-glycol nucleic acid
/sgna-G/	guanosine (S)-glycol nucleic acid
/sgna-T/	thymidine (S)-glycol nucleic acid
/sgna-U/	uridine (S)-glycol nucleic acid
+A	C2'-endo locked adenosine ribonucleotide
+C	C2'-endo locked cytidine ribonucleotide
+G	C2'-endo locked guanosine ribonucleotide
+T	C2'-endo locked thymidine ribonucleotide
+U	C2'-endo locked uridine ribonucleotide
(MOE)A	2'-O-(2-methoxyethyl)adenosine
(MOE)C	2'-O-(2-methoxyethyl)cytidine
(MOE)G	2'-O-(2-methoxyethyl)guanosine
(MOE)U	2'-O-(2-methoxyethyl)uridine
ddA	2',3'-dideoxyadenosine
ddC	2',3'-dideoxycytidine
ddG	2',3'-dideoxyguanosine
ddT	2',3'-dideoxythymidine
ddU	2',3'-dideoxyuridine
VPU	vinylphosphonate 2'O-methoxy uridine
Chd	1-((2S))-1-(2,3-dihydroxypropyl))thymine
Tgn	2'O-hexadecyl cytidine

[0375] List of oligo substrates and products

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
SEQ ID NO:	**Oligonucleotide Sequence**
1063	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mA/32FU/
1064	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FU//i2FC//32FC/
1065	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU*/i2FA/*/i2FA//32FG/
1066	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mA/i2FU/mG/3Phos/
1067	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FU//i2FC//i2FC/mG/3Phos/
1068	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU*/i2FA/*/i2FA//i2FG/mG/3Phos/
1069	/56-FAM/TTTTTTTTTTTTTTTTmC*mA*mGmA
1070	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmAmA/32FG/
1071	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC*mU*mAmC
1072	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG/mU/32FG/
1073	/56-FAM/TTTTTTTTTTTTTTTTmC*mA*mGmAmG/3Phos/
1074	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmAmA/i2FG/mG/3Phos/
1075	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC*mU*mAmCmG/3Phos/
1076	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG/mU/i2FG/mG/3Phos/
1077	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FG/mU/32FC/
1078	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FC/mAmUmC
1079	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FG/mU/i2FC//i2FC//3Phos/
1080	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FC/mAmUmC/i2FC//3Phos/
1081	/56-FAM/TTTTTTTTTTTmU*/i2FA/*/32FA/
1082	/56-FAM/TTTTTTTTTTTTTTTT*/i2FA/*/i2FA//32FG/
1083	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/i2FG/mA/32FU/
1084	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT*/i2FC/*mA/32FA/
1085	/56-FAM/TTTTTTTTTTTmU*/i2FA/*/i2FA//i2FC//3Phos/
1086	/56-FAM/TTTTTTTTTTTTTTTT*/i2FA/*/i2FA//i2FG//i2FC//3Phos/
1087	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/i2FG/mA/i2FU//i2FC//3Phos/
1088	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT*/i2FC/*mA/i2FA//i2FC//3Phos/
1089	/56-FAM/TTTTTTTTTTTTTTTTTTTTmG/i2FU/i2FA/32FC/
1090	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FC/mA/i2FU//32FC/
1091	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA/mUmU/32FG/
1092	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FC/*mA/32FA/
1093	/56-FAM/TTTTTTTTTTTTTTTTTTTTTmG/i2FU/i2FA/i2FC/mA/3Phos/

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
1094	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FC/mA/i2FU//i2FC/mA/3Phos/
1095	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA/mUmU/i2FG/mA/3Phos/
1096	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FC/*mA/i2FA/mA/3Phos/
1097	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTT/mC/i2FC/mC/32FG/
1098	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FC/mG/32FG/
1099	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/mC/i2FC/mC/i2FG//32FC//3Phos/
1100	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FC/mG/i2FG//32FC//3Phos/
1101	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmG/i2FU/mG/32FG/
1102	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmG/i2FU/mG/i2FG/mU/3Phos/
1103	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG/mU/i2FG/mU/3Phos/
1104	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU*/i2FA/*/i2FA//i2FG/mU/3Phos/
1105	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FC/*mA/i2FA/mU/3Phos/
1106	/56-FAM/TTTTTTTTTTTTTTTTTTTTTmG/i2FU/i2FA/i2FC/mG/3Phos/
1107	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FC/mA/i2FU//i2FC/mG/3Phos/
1108	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA/mUmU/i2FG/mG/3Phos/
1109	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FC/*mA/i2FA/mG/3Phos/
1110	/56-FAM/TTTTTTTTTTTTTTTTTTTTTmG/i2FU/i2FA/i2FC/mU/3Phos/
1111	/56-FAM/TTTTTTTTTTTTTTTTmGmUmC*mC
1112	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FG/mU/i2FG/mU
1113	/56-FAM/TTTTTTTTTTTTTTTTTmGmUmC*mC*mA/3Phos/
1114	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/mC/i2FC/mC/i2FG/*mA/3Phos/
1115	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmAmA/i2FG/*mA/3Phos/
1116	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FG/mU/i2FG/mU*mA/3Phos/
1117	/56-FAM/TTTTTTTTTTAmC*mA*mG
1118	/56-FAM/TTTTTTTTTTAmC*mA*mG/i2FU//3Phos/
1119	/56-FAM/TTTTTTTTTTTTTTTmAmUmCmU
1120	/56-FAM/TTTTTTTTTTTTTTTmAmUmCmU/i2FA//3Phos/
1121	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmCmUmU
1122	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmCmUmU/i2FA//3Phos/
1123	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmUmCmU

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
1124	/56-FAM/TTTT

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
1912	/56-FAM/TTTTTTTTTTTTTTTT*/i2FU/*/i2FU//i2FA//32FU/mA/3Phos/
1913	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmG/i2FU/mG/i2FG/mA/3Phos/
1914	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmAmA/32FG/*mG/3Phos/
1915	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mAmU*mA/3Phos/
1916	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA/mUmU/i2FG/mU/3Phos/
1917	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU*/i2FA/*/i2FA//i2FG/mA/3Phos/
1918	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTmAmG/i2FU//i2FG//i2FG/mA/3Phos/
1919	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA//i2FG//i2FU/mG/i2FG//i2FC//3Phos/
1920	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA//i2FG//i2FU/mG/i2FG/mG/3Phos/
1921	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmUmC/i2FC/*mG/3Phos/
1922	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FG/mU/i2FC/mUmU/3Phos/
1923	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmCmUmU/i2FU//3Phos/
1924	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmUmCmU/i2FU//3Phos/
3009	/56-FAM/TTTTTTTTTTTmU*/i2FA/*/i2FG/*/32FA/
3010	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/i2FU//i2FU//i2FC//32FG/
3011	/56-FAM/TTTTTTTTTTTTTTTT*/i2FA/*/i2FG/*/i2FA//32FU/
3012	/56-FAM/TTTTTTTTTTTTTTTTTTTT*mA/i2FA//i2FA//32FA/
3013	/56-FAM/TTTTTTTTTTTmG/i2FU/*mC*/32FA/
3014	/56-FAM/TTTTTTTTTTTTTTTTmGmAmC/32FC/
3015	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FG/*/i2FA/mA/3Phos/
3016	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTT/i2FU//i2FU//i2FC//i2FG/mA/3Phos/
3017	/56-FAM/TTTTTTTTTTTTTTTT*/i2FA/*/i2FG/*/i2FA//i2FU/mA/3Phos/
3018	/56-FAM/TTTTTTTTTTTTTTTTTTTT*mA/i2FA//i2FA//i2FA//i2FA//3Phos/
3019	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FG/*/i2FA//i2FU//3Phos/
3020	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FA/mA/3Phos/
3021	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FA/mU/3Phos/
3022	/56-FAM/TTTTTTTTTTmG/i2FU/*mC*/i2FA/mA/3Phos/
3023	/56-FAM/TTTTTTTTTTmG/i2FU/*mC*/i2FA/mU/3Phos/
3024	/56-FAM/TTTTTTTTTTTTTTTTmGmAmC/i2FC/mU/3Phos/
3025	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FA/mG/3Phos/
3026	/56-FAM/TTTTTTTTTTmU*/i2FA/*/i2FG/*/i2FA//i2FC//3Phos/
	TTTTTTTTmU*/i2FA/*/i2FG/*/i2FA/mA/3Phos/
	TTTTTTTT/i2FU//i2FU//i2FC//i2FG/mA/3Phos/

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
	TTTTTTTT*/i2FA/*/i2FG/*/i2FA//i2FU/mA/3Phos/
	TTTTTTTT*mA/i2FA//i2FA//32FA/
	TTTTTTTTmU*/i2FA/*/i2FG/*/i2FA//i2FU//3Phos/
	TTTTTTTTmU*/i2FA/*/i2FA/mA/3Phos/
	TTTTTTTTmU*/i2FA/*/i2FA/mU/3Phos/
	TTTTTTTTmG/i2FU/*mC*/32FA/
	TTTTTTTTmG/i2FU/*mC*/i2FA/mA/3Phos/
	TTTTTTTTmU*/i2FA/*/i2FG/*/i2FA/mU/3Phos/
	TTTTTTTT/i2FU//i2FU//i2FC//i2FG/mU/3Phos/
	TTTTTTTTmU*/i2FA/*/i2FG/*/32FA/
	TTTTTTTTmU*/i2FA/*/i2FG/*/i2FA/mG/3Phos/
	TTTTTTTTmU*/i2FA/*/i2FG/*/i2FA//i2FC//3Phos/
3027	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC*/i2FG/mA/32FG/
3028	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FG/mUT
3029	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA//i2FA/mG/32FC/
3030	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mU
3031	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mUmA
3032	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC*/i2FG/mA/i2FG/mA/3Phos/
3033	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC*/i2FG/mA/i2FG//i2FA//3Phos/
3034	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FG/mUT/i2FU//3Phos/
3035	/56-FAM/TTTTTTTTTTTTTTTTTmGmAmC/i2FC/mA/3Phos/
3036	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FU//i2FU//i2FC//i2FG/mU/3Phos/
3037	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTT/i2FA//i2FA/mG/i2FC/mA/3Phos/
3038	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mUmA/3Phos/
3039	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mUmG/3Phos/
3040	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mUmA/i2FC//3Phos/
	TTTTTTTT/i2FU//i2FU//i2FC//32FG/
	TTTTTTTTmC*/i2FG/mA/32FG/
	TTTTTTTTmC*/i2FG/mA/i2FG/mA/3Phos/
	TTTTTTTTmC/i2FG/mUT/i2FU//3Phos/
	TTTTTTTTmG/i2FU/i2FA/i2FC/mU/3Phos/
	TTTTTTTT*/i2FA/*/i2FG/*/i2FA//32FU/
	TTTTTTTTmGmAmC/i2FC/mA/3Phos/
	TTTTTTTTmGmAmC/i2FC/mU/3Phos/
	TTTTTTTT/i2FA//i2FA/mG/32FC/
	TTTTTTTT/i2FA//i2FA/mG/i2FC/mU/3Phos/
	TTTTTTTTmA*/i2FG/*mUmA/3Phos/
	TTTTTTTTmA*/i2FG/*mUmG/3Phos/
	TTTTTTTTmA*/i2FG/*mUmA/i2FC//3Phos/
3041	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmA/i2FG/mA
3042	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmUmG/32FU/
3043	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG//i2FG//32FC/
3044	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FG/mUTmA/3Phos/

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
3045	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmG/i2FU/mG/i2FG//i2FA//3Phos/
3046	/56-FAM/TTTTTTTTTTTTTTTTTTTT*mA/i2FA//i2FA//i2FA/mA/3Phos/
3047	/56-FAM/TTTTTTTTTTTTTTTTTTTTTmAmG/i2FA/mG/i2FG/mA/3Phos/
3048	/56-FAM/TTTTTTTTTTTTTTTTTTTTTmAmG/i2FA/mG/i2FG/mU/3Phos/
3049	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmAmA/i2FG/mAmU/3Phos/
3050	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mA/i2FU/mU/3Phos/
3051	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmUmG/i2FU/mA/3Phos/
3052	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mA/i2FU/mA/3Phos/
3053	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG//i2FG//i2FC/mG/3Phos/
3054	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmUmG/i2FU/mG/3Phos/
	TTTTTTTTmUmUmG/32FU/
	TTTTTTTTmG/i2FU/i2FA/i2FC/mA/3Phos/
	TTTTTTTTmC/i2FG/mUTmA/3Phos/
	TTTTTTTT/i2FC/mA/i2FU//i2FC/mA/3Phos/
	TTTTTTTT*mA/i2FA//i2FA//i2FA/mA/3Phos/
	TTTTTTTTmG/i2FU/mG/i2FG/mU/3Phos/
	TTTTTTTmAmG/i2FA/mG/i2FG/mU/3Phos/
	TTTTTTTmAmA/i2FG/mAmU/3Phos/
	TTTTTTTTmU/i2FC/mA/i2FU/mU/3Phos/
	TTTTTTTTmUmUmG/i2FU/mA/3Phos/
	TTTTTTTTmU/i2FC/mA/i2FU/mA/3Phos/
	TTTTTTTTmA/i2FG//i2FG//i2FC/mG/3Phos/
	TTTTTTTTmUmUmG/i2FU/mG/3Phos/
3055	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG//i2FU/mG/32FG/
3056	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA*/i2FG/*mUmAmA/3Phos/
3057	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU*/i2FA/*/i2FA//i2FG//i2FA//3Phos/
3058	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmC/i2FG/mUTmU/3Phos/
3059	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmA/i2FG//i2FU/mG/i2FG/mA/3Phos/
3060	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmUmG/i2FU/mU/3Phos/
3061	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmU/i2FC/mAi2FU//i2FC//3Phos/
	TTTTTTTTmA*/i2FG/*mUmAmA/3Phos/
	TTTTTTTTmAmG/i2FA/mG/i2FG/mA/3Phos/
	TTTTTTTTmC/i2FG/mUTmU/3Phos/
	TTTTTTTTmA/i2FG//i2FU/mG/i2FG/mA/3Phos/
	TTTTTTTTmUmUmG/i2FU/mU/3Phos/
	TTTTTTTT/i2FC/mA/i2FU//i2FC/mG/3Phos/
	TTTTTTTTmU/i2FC/mAi2FU//i2FC//3Phos/
	/56-FAM/TTTTTTTTTTmAmGmG*mG
3062	/56-FAM/TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTmUmCmUmU*mA/3Phos/
	/56-FAM/TTTTTTTTTTmAmGmG*mG*mA/3Phos/

Modified oligonucleotide sequences. Sequences with ten or more native nucleotides have a corresponding SEQ ID NO.	
	TTTTTTTTmAmUmCmU
	TTTTTTTTmAmGmG*mG
	TTTTTTTTmUmCmUmU*mA/3Phos/
	TTTTTTTTmAmGmG*mG*mA/3Phos/
3063	/56-FAM/TTTTTTTTTTTTTTTTTmUmCmCmC
3064	/56-FAM/TTTTTTTTTTTTTTTTTmUmCmCmC*mU/3Phos/
	TTTTTTTTmUmCmCmC
	TTTTTTTTmUmCmCmC*mU/3Phos/
3065	/56-FAM/TTTTTTTTTTTTTTTTT*/i2FU/*/i2FU//i2FA//32FU/
	TTTTTTTT*/i2FU/*/i2FU//i2FA//32FU/
3066	/56-FAM/TTTTTTTTTTTTTTTTT*/i2FU/*/i2FU//i2FA//i2FU/mA/3Phos/
	TTTTTTTT*/i2FU/*/i2FU//i2FA//i2FU/mA/3Phos/
	mA*mA*/i2FG/mC
	mA*/i2FC/*mA/32FA/
	mA*/i2FC/*mA
	mC*mU*mAmG
	mUmCmU
	mU*/i2FU/*mAmG
	mC*mU*mA
	/VPU/*/i2FU/*mA
	mU*/i2FU/*mA
	mU/i2FU/*mU/32FA/
	mA*mA*/i2FG/mC/i2FA//3Phos/
	mA*/i2FC/*mA/i2FA//i2FA//3Phos/
	mA*/i2FC/*mA/i2FA//3Phos/
	mC*mU*mAmGmA/3Phos/
	mUmCmUmA/3Phos/
	mU*/i2FU/*mAmGmG/3Phos/
	mC*mU*mAmG/3Phos/
	/VPU/*/i2FU/*mAmG/3Phos/
	mU*/i2FU/*mAmG/3Phos/
	mU/i2FU/*mU/i2FA/mU/3Phos/
	/VPU/*/i2FU/*mAmG
	/VPU/*/i2FU/*mAmGmGmU/Tgn/mG
	mU*/i2FU/*mAmGmGmU/Tgn/mG
	mG*mG*mCmUmA/Chd/mG
	/VPU/*/i2FU/*mAmGmG/3Phos/
	/VPU/*/i2FU/*mAmGmGmU/Tgn/mGmG/3Phos/
	mU*/i2FU/*mAmGmGmU/Tgn/mGmG/3Phos/
	mG*mG*mCmUmA/Chd/mGmGmA/3Phos/

Example 1

Construction of Expression Vectors

[0376] Evolved terminal nucleotidyl transferase (TnT) enzyme (SEQ ID NO:2, previously engineered as described in PCT/US2023/076667) encoding an *N*-terminal 6-histidine tagged TnT was subcloned into the *E. coli* expression vector pCK100900i (See e.g., US Pat. No. 7,629,157 and US Pat. Appln. Publn. 2016/0244787, both of which are hereby incorporated by reference). This plasmid construct was transformed into an *E. coli* strain derived from W3110. Directed evolution techniques were used to generate libraries of gene variants from these plasmids (See e.g., US Pat. No. 8,383,346 and WO 2010/144103, both of which are hereby incorporated by reference). The substitutions in the enzyme variants described herein are indicated with reference to the N-terminal 6-histidine tagged version of the parent TnT enzyme (i.e., SEQ ID NO: 2) or variants thereof, as indicated.

Example 2

TnT expression and lysate processing for high throughput (HTP) screening

High Throughput (HTP) Growth of TnT Enzyme and Variants

[0377] Transformed *E. coli* cells were selected by plating onto LB agar plates containing 1% glucose and 30 µg/mL chloramphenicol. After overnight incubation at 37 °C, colonies were placed into the wells of 96-well shallow flat bottom NUNC™ (Thermo-Scientific) plates filled with 180 µl/well LB medium supplemented with 1% glucose and 30 µg/mL chloramphenicol. The cultures were allowed to grow overnight for 18-20 hours in a shaker (200 rpm, 30 °C, and 85% relative humidity; Kuhner). Overnight growth samples (20 µL) were transferred into Costar 96-well deep plates filled with 380 µL of Terrific Broth supplemented with 30 µg/mL chloramphenicol. The plates were incubated for 120 minutes in a shaker (250 rpm, 30 C, and 85% relative humidity; Kuhner) until the OD_{600} reached between 0.4-0.8. The cells were then induced with 40 µL of 10 mM IPTG in sterile water and incubated overnight for 18-20 hours in a shaker (250 rpm, 30 C, and 85% relative humidity; Kuhner). The cells were pelleted (4,000 rpm for 20 min), the supernatants were discarded, and the cells were frozen at -80 °C prior to analysis.

Thermal lysis of HTP Cell Pellets with Lysozyme

[0378] For lysis, 400 µL lysis buffer containing 50 mM triethanolamine buffer, pH 7.5, and 0.1 g/L lysozyme were added to the cell pellet in each well. The cells were shaken vigorously at room temperature for 5 minutes on a bench top shaker. A 100-uL aliquot of the re-suspended cells was transferred to a 96-well format 200 µL BioRad PCR plate, then briefly spun-down prior to 1 h heat treatment at the temperature indicated, typically 48-60 °C. Following heat-treatment, the cell debris was pelleted by centrifugation (4,000 rpm at 4 °C for 10 min), and clear supernatants were then used in biocatalytic reactions to determine their activity levels.

Example 3

Shake Flask Expression and Purification of TnT

Shake Flask Expression

[0379] Variants described above were streaked out onto agar plates containing 30 µg/mL chloramphenicol and 0.01% glucose (*v/v*) for single colonies. Then, a single colony was used to inoculate a 5-mL tube

containing Terrific Broth (TB) media (Teknova) and 30 μg/mL chloramphenicol, which was then shaken for 20 hours at 30°C, 250 rpm. A glycerol stock was then prepared by mixing the overnight culture with 50% glycerol at a 2:1 ratio. These cultures grown, were thawed, and 30 μL from each glycerol stock was used to inoculate 1 L shake flasks containing: 160 mL Terrific Broth (TB) media (Teknova), 30 μg/mL chloramphenicol, 0.03% (*v/v*) lactose and 0.075% (*v/v*) glucose. The shake flasks were grown at 32 °C for 18 hours at 250 rpm. Following this incubation period, the cultures were centrifuged at 4,000 rpm for 10 min at 4 °C. The culture supernatant was discarded, and the pellets were resuspended in 30 mL of 50 mM Tris-HCl, pH 7.5. The cell suspension was chilled in an ice bath and lysed using a Microfluidizer cell disruptor (Microfluidics M-110L). The crude lysate was pelleted by centrifugation (10,000 rpm for 90 min at 4 °C) in an Avanti J-series centrifuge and JLA-16.250 fixed rotor (Beckman Coulter). The supernatant was then filtered through a 0.2 μm PES membrane to further clarify the lysate. The clarified lysates were then supplemented with 20 mM imidazole and 500 mM NaCl.

Purification of TnT from Shake Flask Lysates

[0380] TnT lysates were supplemented with 1/10th volume of SF elution buffer (50 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, 0.02% *v/v* Triton X-100 reagent) per well. Lysates were then purified using an AKTA Pure purification system and a 5 mL HisTrap FF column (GE Healthcare). The run parameters are given in Table 3.1. The SF wash buffer comprised 50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 0.02% v/v Triton X-100 reagent.

Table 3.1 Purification Parameters

Parameter	Volume
Column volume	5 mL
Wash flow rate	12 mL/min
Elution flow rate	5 mL/min
Pressure limit	0.3 MPa
Sample volume	40 mL
Equilibration volume	5 column volumes (CV) = 25 mL
Wash Unbound volume	20 CV = 100 mL
Elution	Isocratic (step)
Elution volume	5 CV = 25 mL
Fraction volume	1.5 mL
RE-equilibration volume	5 CV = 25 mL

[0381] Elution fractions containing protein were identified by UV absorption (A280) and pooled, then dialyzed overnight in dialysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, and 50% glycerol) in a 3.5K Slide-A-Lyzer™ dialysis cassette (Thermo Fisher) for buffer exchange. TnT concentrations in the preparations were measured by absorption at 280 nm.

Example 4

Capillary electrophoresis (CE) analysis of oligonucleotides

Sample preparation for reaction analysis using CE:

[0382] For analysis of the reaction samples, capillary electrophoresis was performed using an ABI 3500xl Genetic Analyzer (ThermoFisher). Reactions (20 μL) were quenched by the addition of 60 μL of 35 mM

aqueous EDTA. Reactions (1 µL) were quenched by the addition of 99 µL of 1 mM aqueous EDTA. Quenched reactions were diluted in water to 1.25 nM oligonucleotide, and a 2-µL aliquot of this solution was transferred to a new 96-well MicroAmp Optical PCR plate or 384-well MicroAmp Optical PCR plate containing 18 µL Hi-Di™ Formamide (ThermoFisher) containing an appropriate size standard (LIZ or Alexa633). The ABI3500xl was configured with POP6 polymer, 50 cm capillaries, and a 55 °C oven temperature. Pre-run settings were 18KV for 50 sec. Injection was 10KV for 2 sec, and the run settings were 19KV for 620 sec. FAM-labeled oligo substrates and products were identified by their sizes relative to the sizing ladder.

Example 5

Improvements over SEQ ID NO: 2 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0383] SEQ ID NO: 2 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 5.1**.

[0384] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 5.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol %): 25; Pre-incubation Temp (°C): 46; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 46; Reaction Time (min): 120; Reaction volume (µL): 1; NTP: fUTP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T11AmC*mA*mG, dA6AmC*mAmG; Concentration oligo (µM): 50 (FAM label), 450 (no label); Product: 5'-6-FAM-T11AmC*mA*mGfU-3'P, dA6AmC*mAmGfU-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 1.

[0385] Activity relative to SEQ ID NO: 2 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 5.2**.

Table 5.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 2		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2)**	**FIOP % product relative to SEQ ID NO: 2**
3/4	K451R	+++
5/6	K451R/R477Q	+++
7/8	L296V/K451R/R477Q	+++
9/10	K384S/K451R/R477S	++
11/12	A415P/K451R/R477Q/R480W/I503R	++
13/14	L296V/K384S/D412M/A415P/K451R/R477Q/R480W	++
15/16	P404A	++
17/18	L296V/A415P/K451R/R477Q/R480W	++
19/20	R477Q	++
21/22	G402K/P404A/G460S	+
23/24	L296V/K451R/R480W	+
25/26	K384A/D412M/K451R/R480W	+
27/28	E233P/G402K	+
29/30	G402K/P404A/G460A	+
31/32	P404A/S431G	+
Levels of increased activity were determined FIOP % product relative to SEQ ID NO: 2 and are defined as follows: "+" > 1.09, "++" > 1.12, "+++" > 1.17.		

Example 6

Improvements over SEQ ID NO: 8 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0386] SEQ ID NO: 8 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 6.1**.

[0387] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 6.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 60; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: fATP-3'P; NTP conc (µM): 200; Oligonucleotide: 5'-6-FAM-T15mAmUmCmU, T15mAmUmCmU; Concentration oligo (µM): 50 (FAM label), 50 (no label); Product: 5'-6-FAM-T15mAmUmCmUfA-3'P, T15mAmUmCmUfA-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 1.0.

[0388] Activity relative to SEQ ID NO: 8 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 8 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 6.2**.

Table 6.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 8		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 8)**	**Pos Control FIOP % product relative to SEQ ID NO: 8**
33/34	V273R/D277T/L381Y/D492M	+++
35/36	V273R/D277S/L381Y	+++
37/38	V273R/F462M/D492S	+++
39/40	R92C/V273R/D277T/F462M	+++
41/42	V273R/D277S/R429A/D492S	+++
43/44	V273R/D277S/L381Y/R429A	+++
45/46	D277T/L381Y/D492S	+++
47/48	V273R/D277S/L381Y/F462M/D492Y	+++
49/50	D277S/D492S	+++
51/52	V273R/D277S	+++
53/54	V273R/D277T	+++
55/56	D277T/L381Y/D492M	+++
57/58	V273R/F462M/D492Y	++
59/60	V273R/D277S/L381Y/F462M/D492M	++
61/62	V273R/D277S/L381Y/F462M	++
63/64	V273R/D277S/F462M	++
65/66	V273R/D277T/F462M	++
67/68	V273R/L381Y/D492M	++
69/70	V273R/D277S/L381Y/P404S/F462M/D492M	++
71/72	D277S/L381Y/F462M/D492S	++
73/74	V273R/D277T/R429A	++
75/76	Y309H/A313S/R314A/D412N/E485L	++
77/78	D277S/R429A/D492Y	++
79/80	V273R/L381Y	+
81/82	D277S/R429A/F462M/D492S	+
83/84	R267S/A313S/R314A/A408V/E485L	+
85/86	D277T/L381Y	+
87/88	R267S/E367S/E485L	+
89/90	V273R/D277T/L381Y/R429A	+
91/92	D277T/L381Y/R429A/F462M/D492M	+
93/94	R267S/R314A/A408V/M441E	+
95/96	D277S/L381Y	+
97/98	D277S/L381Y/R429A/D492M	+
99/100	L381Y/F462M/D492Y	+
101/102	D277S/L381Y/R429A/F462M/D492M	+
103/104	R267S/A313S/G406H	+
105/106	L381Y/D492S	+

Table 6.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 8		
107/108	R267S/G406H/A408V/E485L	+
109/110	R267S/A313T/R314A	+
111/112	V273R/D277T/L381Y	+
113/114	R267S/A313S/E367S/E485L	+
115/116	V273R/D277S/R429A	+
117/118	D277S/L381Y/F462M/D492M	+
119/120	V273R/F462M	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 8 and are defined as follows: "+" > 4.93, "++" > 7.0, "+++" > 7.75.		

Example 7

Improvements over SEQ ID NO: 8 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0389] SEQ ID NO:8 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 7.1**.

[0390] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 7.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 20; Pre-incubation Temp (°C): 63; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 100; Reaction Time (min): 60; Reaction volume (µL): 1.25; NTP: fATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T15mAmUmCmU, T8mAmUmCmU; Concentration oligo (µM): 100 (FAM label), 400 (no label); Product: 5'-6-FAM-T15mAmUmCmUfA-3'P, T8mAmUmCmUfA-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 2.

[0391] Activity relative to SEQ ID NO: 8 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 8 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 7.2**.

Table 7.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 8		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 8)**	**Pos Control FIOP % product relative to SEQ ID NO: 8**
121/122	D198R	+++
123/124	G274R	+++
125/126	D198S	+++
127/128	G196K	+++
129/130	E186R	++
131/132	D198R/E515G	++
133/134	D198H	++
135/136	V193W	++
137/138	V193T	+
139/140	Y394S	+
141/142	V193Q	+
143/144	D198Q	+
145/146	D409R	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 8 and are defined as follows: "+" > 2.05, "++" > 4.87, "+++" > 7.55.		

Example 8

Improvements over SEQ ID NO: 70 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0392] SEQ ID NO: 70 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 8.1**.

[0393] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 8.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 46; Pre-incubation Time (min): 60; Reaction temperature (°C): 46; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: mGTP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T10mCmCmUfA, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T51mU*fA*fAfG; Concentration oligo (µM): 125; Product: 5'-6-FAM-T10mCmCmUfAmG-3'P, 5'-6-FAM-T31mUfCmAfUmG-3'P, 5'-6-FAM-T41mAfUfCfCmG-3'P, 5'-6-FAM-T51mU*fA*fAfGmG-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 1.

[0394] Activity relative to SEQ ID NO: 70 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 70 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 8.2**.

Table 8.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 70

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 70)	Pos Control FIOP % product relative to SEQ ID NO: 70
147/148	E190R/R267S/G274P	+++
149/150	G274P	+++
151/152	I208E/G274P/Y309H/A313S/K430Q	+++
153/154	E190R/I208G/R267S/G274P/Y309H/C417A	+++
155/156	E190A/G274P/C417A	++
157/158	G274P/Y309H/C417A/Q477R	++
159/160	G274P/Y309H/A313S/C417A/M462H	++
161/162	E190A/I208E/R267S/G274P	++
163/164	G274P/Y309H	++
165/166	G274P/A313S	+
167/168	R273V	+
169/170	E190A/I208E/Y309H/M462H	+
171/172	E190A	+
173/174	E190A/I208G/R267S/G274P/C417A/M462H	+
175/176	R267S/G274P/Q477R	+
177/178	E190R/G274P/C417A/Q477R	+
179/180	E190R/I208G/G274P/Y309H/A313S/C417A/M462H	+
181/182	G274P/M462H	+

Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 70 and are defined as follows: "+" > 1.23, "++" > 1.36, "+++" > 1.59.

Example 9

Improvements over SEQ ID NO: 148 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0395] SEQ ID NO: 148 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 9.1**.

[0396] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 9.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 5; Pre-incubation Temp (°C): 46; Pre-incubation Time (min): 60; Reaction temperature (°C): 46; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: mGTP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T46mAfGmUfG; Concentration oligo (µM): 125; Product: 5'-6-FAM-T16mC*mA*mGmAmG-3'P, 5'-6-FAM-T31mAmAmAfGmG-3'P, 5'-6-FAM-T36mC*mU*mAmCmG-3'P, 5'-6-FAM-T46mAfGmUfGmG-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 2.

[0397] Activity relative to SEQ ID NO: 148 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 148 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 9.2**.

Table 9.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 148		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 148)**	**Pos Control FIOP % product relative to SEQ ID NO: 148**
183/184	I208E/I343L/K407P	++
185/186	I208D/I343L/K407P	++
187/188	D183R/I208E/Y309H/A313S/K407P/M462H	+
189/190	I208E/K407P	+
191/192	Y309H	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 148 and are defined as follows: "+" > 1.09, "++" > 1.14.		

Example 10

Improvements over SEQ ID NO: 148 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0398] SEQ ID NO: 148 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 10.1**.

[0399] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 10.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T36fCmAmUmC, , ; Concentration oligo (µM): 100 (each with label), 150 (each w/o label); Product: 5'-6-FAM-T26mUfGmUfCfC-3'P, 5'-6-FAM-T36fCmAmUmCfC-3'P, , ; IPP: SEQ ID NO: 1060; IPP (µM): 1.

[0400] Activity relative to SEQ ID NO: 148 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 148 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 10.2**.

Table 10.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 148		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 148)**	**Pos Control FIOP % product relative to SEQ ID NO: 148**
193/194	R190H	+++
195/196	A415P	+++
197/198	Q477R	++
199/200	C417S	++
201/202	K303T	++
203/204	D409P	+
205/206	A415G	+
207/208	V268L	+
209/210	T275L	+
211/212	Q397V	+
213/214	T275S	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 148 and are defined as follows: "+" > 1.05, "++" > 1.07, "+++" > 1.1.		

Example 11

Improvements over SEQ ID NO: 148 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0401] SEQ ID NO: 148 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 11.1**.

[0402] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a

peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 11.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 64; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration oligo (µM): 100 (FAM label), 400 (no label); Product: 5'-6-FAM-T46mUmCmUmUfA-3'P, T8mUmCmUmUfA-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 1.0.

[0403] Activity relative to SEQ ID NO: 148 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 148 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 11.2**.

Table 11.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 148		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 148)**	**Pos Control FIOP % product relative to SEQ ID NO: 148**
199/200	C417S	++
203/204	D409P	+
215/216	E186T	+++
217/218	I420L	+++
219/220	E186V	+++
221/222	R318L	++
223/224	Q353L	++
225/226	V304L	+
227/228	Q347A	+
229/230	S416H	+
231/232	E186L	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 148 and are defined as follows: "+" > 1.14, "++" > 1.19, "+++" > 1.25.		

Example 12

Improvements over SEQ ID NO: 184 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0404] SEQ ID NO: 184 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 12.1**.

[0405] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000

μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 12.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 5; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (μL): 1; NTP: fCTP-3'P; NTP conc (μM): 600; Oligonucleotide: 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T41*fC*mAfA; Concentration each oligo (μM): 125; Product: 5'-6-FAM-T11mU*fA*fAfC-3'P, 5'-6-FAM-T17*fA*fAfGfC-3'P, 5'-6-FAM-T27fGmAfUfC-3'P, 5'-6-FAM-T41*fC*mAfAfC-3'P; IPP: SEQ ID NO: 1058; IPP (μM): 2.

[0406] Activity relative to SEQ ID NO: 184 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 184 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 12.2**.

Table 12.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 184		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 184)**	**Pos Control FIOP % product relative to SEQ ID NO: 184**
233/234	V193S/A465G	+++
235/236	P274N/P352M/Q353T/A465G	+++
237/238	Q281S/G411T/A465G	+++
239/240	V193S/P274N/G411T/A465G/F525H	++
241/242	V193S/A408L/A465G	++
243/244	L343I/G411T	++
245/246	G411T	+
247/248	L343I/A408L/A465G	+
249/250	G411T/F525H	+
251/252	F525H	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 184 and are defined as follows: "+" > 1.14, "++" > 1.18, "+++" > 1.27.		

Example 13

Improvements over SEQ ID NO: 234 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0407] SEQ ID NO: 234 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 13.1**.

[0408] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 13.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 20; Pre-incubation Temp (°C): 66; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (µL): 1.25; NTP: fATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T15mAmUmCmU, T8mAmUmCmU; Concentration each oligo (µM): 100 (FAM label), 400 (no label); Product: 5'-6-FAM-T15mAmUmCmCfA-3'P, T8mAmUmCmCfA-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 2.

[0409] Activity relative to SEQ ID NO: 234 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 234 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 13.2**.

Table 13.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 234)	Pos Control FIOP % product relative to SEQ ID NO: 234
253/254	T300K	+++
255/256	Q397K	+++
257/258	Q397I	+++
259/260	V393Y	+++
261/262	T300F	+++
263/264	Q397W	++
265/266	T300M	++
267/268	T300R	++
269/270	Q397F	++
271/272	T299R	++
273/274	T300G	++
275/276	T300I	++
277/278	T300N	++
279/280	T300L	++
281/282	T299S	++
283/284	K392H	+
285/286	Q397Y	+
287/288	V393G	+
289/290	K392N	+
291/292	Q397M	+
293/294	T300Y	+
295/296	T299G	+

Table 13.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234		
297/298	Q397R	+
299/300	Q397V	+
301/302	Q397H	+
303/304	T299P	+
305/306	T300Q	+
307/308	T275I	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 234 and are defined as follows: "+" > 1.96, "++" > 3.85, "+++" > 6.65.		

Example 14

Improvements over SEQ ID NO: 234 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0410] SEQ ID NO: 234 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 14.1**.

[0411] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 14.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.125; NTP: mATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T21mGfUfAfCmA-3'P, 5'-6-FAM-T36fCmAfUfCmA-3'P, 5'-6-FAM-T46fAmUmUfGmA-3'P, 5'-6-FAM-T56mA*fC*mAfAmA-3'P; IPP: SEQ ID NO: 1058; IPP (µM): 2.

[0412] Activity relative to SEQ ID NO: 234 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 234 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 14.2**.

Table 14.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 234)**	**Pos Control FIOP % product relative to SEQ ID NO: 234**
261/262	T300F	+++
265/266	T300M	++
271/272	T299R	+
277/278	T300N	+
279/280	T300L	++
281/282	T299S	+++
289/290	K392N	+++
291/292	Q397M	++
293/294	T300Y	++
295/296	T299G	++
309/310	K392S	+++
311/312	K392Q	+++
313/314	W395F	+++
315/316	T300V	++
317/318	W395L	++
319/320	V393L	++
321/322	T300S	++
323/324	T299N	++
325/326	V393M	++
327/328	T300E	++
329/330	W395M	++
331/332	V393T	++
333/334	V393I	+
335/336	V393A	+
337/338	W395T	+
339/340	Q397E	+
341/342	W395Q	+
343/344	T300A	+
345/346	W395I	+
347/348	Q397L	+
349/350	K392E	+
351/352	V393S	+
353/354	T299A	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 234 and are defined as follows: "+" > 1.02, "++" > 1.05, "+++" > 1.08.		

Example 15

Improvements over SEQ ID NO: 234 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0413] SEQ ID NO: 234 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 15.1**.

[0414] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT,

were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 15.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 20; Pre-incubation Temp (°C): 50; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 1; NTP: fCTP-3'P; NTP conc (µM): 1000; Oligonucleotide: 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T36mCfCmGfG; Concentration each oligo (µM): 100 (each label), 150 (each unlabeled); Product: 5'-6-FAM-T26mCfCmCfGfC-3'P, 5'-6-FAM-T36mCfCmGfGfC-3'P; IPP: SEQ ID NO: 1060; IPP (µM): 1.

[0415] Activity relative to SEQ ID NO: 234 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 234 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 15.2**.

Table 15.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 234)**	**Pos Control FIOP % product relative to SEQ ID NO: 234**
355/356	I420V	++
357/358	S405T	++
359/360	L359F	++
361/362	F525T	++
363/364	T307S	+
365/366	M492H	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 234 and are defined as follows: "+" > 1.03, "++" > 1.06.		

Example 16

Improvements over SEQ ID NO: 234 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0416] SEQ ID NO: 234 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 16.1**.

[0417] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT,

were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 16.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 64.5; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 120; Reaction volume (µL): 1; NTP: fATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T41mAmUmCmU, T8mAmUmCmU; Concentration each oligo (µM): 100 (FAM label), 400 (no label); Product: 5'-6-FAM-T41mAmUmCmUfA-3'P, T8mAmUmCmUfA-3'P; IPP: SEQ ID NO: 1060; IPP (µM): 1.0.

[0418] Activity relative to SEQ ID NO: 234 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 234 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 16.2**.

Table 16.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 234)**	**Pos Control FIOP % product relative to SEQ ID NO: 234**
365/366	M492H	++
367/368	Y394K	+++
369/370	W382M	+++
371/372	P458K	+++
373/374	M492G	+++
375/376	M492R	+++
377/378	N493R	+++
379/380	S404I	+++
381/382	M492S	+++
383/384	Q468I	+++
385/386	D409R	+++
387/388	D409G	+++
389/390	M466L	++
391/392	K392R	++
393/394	Q468H	++
395/396	V328I	++
397/398	D412V	++
399/400	S495L	++
401/402	M492C	++
403/404	L419M	++
405/406	D409Y	++
407/408	E414R	++
409/410	S495G	++
411/412	G411H	++
413/414	D409S	++
415/416	R318I	++

Table 16.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 234		
417/418	E410V	++
419/420	S404L	++
421/422	I361L	++
423/424	C390I	++
425/426	L298I	+
427/428	W398M	+
429/430	G411R	+
431/432	L312M	+
433/434	F525I	+
435/436	S404R	+
437/438	D322A	+
439/440	L359V	+
441/442	A319M	+
443/444	I513V	+
445/446	S495T	+
447/448	H494Q	+
449/450	C417G	+
451/452	C457T	+
453/454	A415S	+
455/456	Q468M	+
457/458	Y308L	+
459/460	M379T	+
461/462	S405Y	+
463/464	L517I	+
465/466	G411V	+
467/468	I420C	+
469/470	A319T	+
471/472	L419I	+
473/474	L389A	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 234 and are defined as follows: "+" > 1.73, "++" > 2.91, "+++" > 5.11.		

Example 17

Improvements over SEQ ID NO: 256 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0419] SEQ ID NO: 256 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 17.1**.

[0420] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 17.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mUTP-3'P; NTP conc (µM): 1000; Oligonucleotide: 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T41mGfUmGfGmU-3'P, 5'-6-FAM-T46mAfGmUfGmU-3'P, 5'-6-FAM-T51mU*fA*fAfGmU-3'P, 5'-6-FAM-T56mA*fC*mAfAmU-3'P; IPP: SEQ ID NO: 1060; IPP (µM): 1.0.

[0421] Activity relative to SEQ ID NO: 256 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 256 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 17.2**.

Table 17.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 256		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 256)**	**Pos Control FIOP % product relative to SEQ ID NO: 256**
475/476	A415P	+++
477/478	A319T/M492H/S495G	+++
479/480	T307S	+++
481/482	W325G/L359F/S404T/D409R	++
483/484	S405T/D409R	++
485/486	A319T/E410V/S495G	++
487/488	F525T	++
489/490	L359F/S404R/S405T	+
491/492	D412V	+
493/494	E410V	+
495/496	G442R/C457T	+
497/498	L419M	+
499/500	S405T	+
501/502	A319T/M466L/M492H/S495G	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 256 and are defined as follows: "+" > 1.19, "++" > 1.34, "+++" > 1.75.		

Example 18

Improvements over SEQ ID NO: 256 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0422] SEQ ID NO: 256 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 18.1**.

[0423] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 18.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 65.5; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0424] Activity relative to SEQ ID NO: 256 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 256 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 18.2**.

Table 18.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 256

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 256)	Pos Control FIOP % product relative to SEQ ID NO: 256
503/504	S439L	+++
505/506	M435L	+++
507/508	A339T	+++
509/510	G402D	+++
511/512	S363P	+++
513/514	G402S	+++
515/516	R501L	+++
517/518	R380L	+++
519/520	E443A	++
521/522	R380A	++
523/524	K384G	++
525/526	S363P/N434P	++
527/528	M499R	++
529/530	P436L	++
531/532	D336C	++
533/534	R380V	++
535/536	G402T	++
537/538	M441T	++
539/540	K432V	++
541/542	D336T	++
543/544	K368S	++

Table 18.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 256		
545/546	P506L	++
547/548	K430S	++
549/550	M435T	++
551/552	S362M	++
553/554	G460S	++
555/556	Y497L	+
557/558	T437A	+
559/560	G460V	+
561/562	K384T	+
563/564	A323T	+
565/566	P436C	+
567/568	Q504P	+
569/570	P506A	+
571/572	G442L	+
573/574	R380S	+
575/576	G460R	+
577/578	V428A	+
579/580	L403I	+
581/582	H433T	+
583/584	N440M	+
585/586	G438P	+
587/588	R459L	+
589/590	P436H	+
591/592	N434P	+
593/594	N434L	+
595/596	N434G	+
597/598	K432I	+
599/600	S439P	+
601/602	H426G	+
603/604	K383L	+
605/606	S439C	+
607/608	T437P	+
609/610	M435F	+
611/612	E443V	+
613/614	E443P	+
Levels of increased activity were determined pos Control FlOP % product relative to SEQ ID NO: 256 and are defined as follows: "+" > 1.38, "++" > 1.7, "+++" > 2.31.		

Example 19

Improvements over SEQ ID NO: 256 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0425] SEQ ID NO: 256 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 19.1**.

[0426] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 μM oligonucleotide, 600-1000 μM nucleotide triphosphate, 1-2 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT,

were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 19.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: fCTP-3'P; NTP conc (µM): 1500; Oligonucleotide: 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T21mGfUfAfCmG-3'P, 5'-6-FAM-T36fCmAfUfCmG-3'P, 5'-6-FAM-T46fAmUmUfGmG-3'P, 5'-6-FAM-T56mA*fC*mAfAmG-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0427] Activity relative to SEQ ID NO: 256 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 256 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 19.2**.

Table 19.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 256		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 256)**	**Pos Control FIOP % product relative to SEQ ID NO: 256**
507/508	A339T	++
509/510	G402D	+++
513/514	G402S	+
515/516	R501L	+
517/518	R380L	+
521/522	R380A	+
525/526	S363P/N434P	+++
531/532	D336C	+
533/534	R380V	++
535/536	G402T	+++
543/544	K368S	+
545/546	P506L	++
549/550	M435T	+
553/554	G460S	++
555/556	Y497L	+
557/558	T437A	++
559/560	G460V	+++
561/562	K384T	+
565/566	P436C	++
569/570	P506A	+++
585/586	G438P	++
587/588	R459L	+
597/598	K432I	+
599/600	S439P	+
611/612	E443V	++

Table 19.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 256		
615/616	K383Q	+++
617/618	K397T	+++
619/620	K368L	+++
621/622	G402M	++
623/624	G460L	++
625/626	K430W	++
627/628	G518S	++
629/630	K508L	++
631/632	G402A	++
633/634	G402P	+
635/636	A339I	+
637/638	H433V	+
639/640	L427A	+
641/642	Y381S	+
643/644	Q385V	+
645/646	M499L	+
647/648	P436G	+
649/650	V428T	+
651/652	S363T	+
653/654	Q329H	+
655/656	S362A	+
657/658	D425R	+
659/660	T500A	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 256 and are defined as follows: "+" > 1.14, "++" > 1.3, "+++" > 1.4.		

Example 20

Improvements over SEQ ID NO: 478 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0428] SEQ ID NO: 478 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 20.1**.

[0429] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 20.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 1; NTP: mUTP-3'P; NTP conc (µM): 1500; Oligonucleotide: 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T21mGfUfAfCmU-3'P, 5'-6-FAM-T41mGfUmGfGmU-3'P, 5'-6-FAM-T51mU*fA*fAfGmU-3'P, 5'-6-FAM-T56mA*fC*mAfAmU-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0430] Activity relative to SEQ ID NO: 478 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 478 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 20.2**.

Table 20.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 478		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 478)**	**Pos Control FIOP % product relative to SEQ ID NO: 478**
661/662	T299S/R459L	+++
663/664	R459L	+++
665/666	G402S/S416A/R459L	++
667/668	T299S/S363P/G402S	++
669/670	A302S/G402S/R459L	++
671/672	T299S/S363P/S405T/S416A	++
673/674	T299S/A302S/S363P/G402S/S405T/S416A	+
675/676	T299S/G402S/S416A/R501L	+
677/678	A302S/S363P/G402S/S405T	+
679/680	T299S/S363P/R459L	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 478 and are defined as follows: "+" > 1.15, "++" > 1.27, "+++" > 1.4.		

Example 21

Improvements over SEQ ID NO: 668 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0431] SEQ ID NO: 668 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 21.1**.

[0432] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a

peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 21.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 48; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 1; NTP: mUTP-3'P; NTP conc (µM): 1500; Oligonucleotide: 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T21mGfUfAfCmU-3'P, 5'-6-FAM-T41mGfUmGfGmU-3'P, 5'-6-FAM-T51mU*fA*fAfGmU-3'P, 5'-6-FAM-T56mA*fC*mAfAmU-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0433] Activity of the variant relative to SEQ ID NO: 668 (Activity FIOP) was calculated as the percent of one of the products from the multiplexed assay, 5'-6-FAM-T21mGfUfAfCmU-3'P, defined as the sum of the area of the indicated product divided by the sum of the total peak area, compared with the percent of the product observed by the reaction with SEQ ID NO: 668 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 21.2**.

Table 21.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 668		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 668)**	**Pos Control FIOP % product 5'-6-FAM-T21mGfUfAfCmU-3'P relative to SEQ ID NO: 668**
681/682	S416A	++
683/684	A302S/S416A/Y497L	++
685/686	K368S/S416A/M435L/P436H/R501L	+
687/688	K368S	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 668 and are defined as follows: "+" > 1.38, "++" > 2.0.		

Example 22

Improvements over SEQ ID NO: 668 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

Screening of shake-flask purified TnT variants for improved activity.

[0434] TnT variants SEQ ID NO: 668, 682, and 690 were produced in shake flask and purified as described in Example 3.

[0435] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 2500 µM oligonucleotide, 3000 µM nucleotide triphosphate, 5 µM TnT, 1 µM IPP SEQ ID NO: 1062, 100 mM triethanolamine (pH 7.8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature

and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 22.1
Reaction buffer-100 mM TEA, 1 mM $CoCl_2$, pH 7.8; IPP SEQ ID NO: 1062 (μM)- 1; Reaction temperature (C)- 50; Reaction Time (min)- 60 min; Reaction volume (μL)- 1; TnT concentration (μM)- 5; NTP- fCTP-3'P; NTP conc (μM)-3000; Oligonucleotide- 5'-6-FAM-T41mGfUmGfG, T8mGfUmGfG ; Oligonucleotide conc (μM)-100 (labeled), 2400 (unlabeled) ; Products- 5'-6-FAM-T41mGfUmGfGfC-3'P, T8mGfUmGfGfC-3'P.

[0436] Activity relative to SEQ ID NO: 668 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 668 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 22.2**.

Table 22.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 668		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 668)**	**Pos Control FIOP % product relative to SEQ ID NO: 668**
681/682	S416A	+
689/690	A339T/K368S/P436L	+
Levels of increased activity were determined FIOP % product relative to SEQ ID NO: 668 and are defined as follows: "+" > 1.04.		

Example 23

Improvements over SEQ ID NO: 668 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0437] SEQ ID NO: 668 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 23.1**.

[0438] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 μM oligonucleotide, 600-1000 μM nucleotide triphosphate, 1-2 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 23.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 25; Pre-incubation Temp (°C): 65.5; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T7mUmCmUmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T7mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0439] Activity relative to SEQ ID NO: 668 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 668 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 23.2**.

Table 23.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 668		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 668)**	**Pos Control FIOP % product relative to SEQ ID NO: 668**
691/692	E246V	+++
693/694	S444G	+++
695/696	M490I	+++
697/698	G236S	+++
699/700	N191F	+++
701/702	T319G	++
703/704	S184R	++
705/706	E288D	++
707/708	N191Y	++
709/710	D232E	++
711/712	T319W	++
713/714	K222A	+
715/716	E485I	+
717/718	Q251R	+
719/720	N191W	+
721/722	R291K	+
723/724	T224L	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 668 and are defined as follows: "+" > 1.0, "++" > 1.07, "+++" > 1.19.		

Example 24

Improvements over SEQ ID NO: 682 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

Screening of shake-flask purified TnT variants for improved activity.

[0440] TnT variants SEQ ID NO: 682, 726, 728, 730, and 732 were produced in shake flask and purified as described in Example 3.

[0441] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 2500 µM oligonucleotide, 3000 µM nucleotide triphosphate, 5 µM TnT, 1 µM IPP SEQ ID NO: 1062, 100 mM triethanolamine (pH 7.8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all

reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 24.1
Reaction buffer-100 mM TEA, 1 mM $CoCl_2$, pH 7.8; IPP SEQ ID NO: 1062 (μM)- 1; Reaction temperature (C)- 50; Reaction Time (min)- 60 min; Reaction volume (μL)- 1; TnT concentration (μM)- 5; NTP- mGTP-3'P; NTP conc (μM)-3000; Oligonucleotide- 5'-6-FAM-T41mGfUmGfG, T8mGfUmGfG ; Oligonucleotide conc (μM)-100 (labeled), 2400 (unlabeled) ; Products- 5'-6-FAM-T41mGfUmGfGmG-3'P, T8mGfUmGfGmG-3'P.

[0442] Activity relative to SEQ ID NO: 682 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 682 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 24.2**.

Table 24.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 682		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 682)**	**Pos Control FIOP % product relative to SEQ ID NO: 682**
725/726	K368S	+
727/728	N191F	++
729/730	E485I	++
731/732	M490I	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 682 and are defined as follows: "+" > 1.01, "++" > 1.04.		

Example 25

Improvements over SEQ ID NO: 730 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0443] SEQ ID NO: 730 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 25.1**.

[0444] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 μM oligonucleotide, 600-1000 μM nucleotide triphosphate, 1-2 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 25.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 65; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60; Reaction volume (µL): 1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0445] Activity relative to SEQ ID NO: 730 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 730 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 25.2**.

Table 25.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 730		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 730)**	**Pos Control FIOP % product relative to SEQ ID NO: 730**
733/734	K222A/E246V/K368S/S444G	+++
735/736	N191F/K368S/M490V	+++
737/738	N191F/R291K/K368S/S444G	+++
739/740	N191F/K368S/S444G	+++
741/742	N191F/K222A/K368S/S444G/M490V	+++
743/744	K222A/K368S/S444G/M490I	+++
745/746	K222A/V244R/R291K/K368S/S444G	+++
747/748	K368S/S444G	+++
749/750	G236S/E246V/R291K/K368S/S444G/M490V	+++
751/752	E246V/R291K/K368S/M490V	+++
753/754	G236S/E246V/K368S	++
755/756	N191F/K222A/V244R/E246V/R291K/K368S/M490I	++
757/758	N191F/E246V/R291K/K368S/M490I	++
759/760	E246V/R291K/K368S	++
761/762	K222A/K368S/S444G/M490V	++
763/764	K222A/R291K/K368S/S444G/M490V	++
765/766	N191F/K222A/K368S/M490I	++
767/768	N191F/K222A/V244R/R291K/K368S/S444G	++
769/770	N191F/K222A/R291K/K368S	++
771/772	N191F/K222A/G236S/E246V/R291K/K368S/S444G	++
773/774	E246V/K368S/S444G	+
775/776	N191F/K222A/E246V/K368S/S444G	+
777/778	N191F/K222A/R291K/K368S/M490V	+
779/780	V244R/R291K/K368S/M490V	+
781/782	N191F/E246V/K368S/M490V	+
783/784	N191F/G236S/R291K/K368S	+
785/786	K222A/R291K/K368S	+
787/788	K222A/G236S/E246V/R291K/K368S/S444G/M490V	+
789/790	K222A/R291K/K368S/S444G	+
791/792	K222A/K368S/M490V	+
793/794	N191F/K368S/S444G/M490V	+

Table 25.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 730		
795/796	V244R/E246V/R291K/K368S	+
797/798	N191F/G236S/V244R/R291K/K368S/M490V	+
799/800	K222A/E246V/M490I	+
801/802	N191F/K222A/V244R/E246V/K368S/G495S	+
803/804	N191F/K222A/V244R/E246V/K368S/M490I	+
805/806	N191F/K222A/K368S	+
807/808	K368S	+
809/810	K368S/M490V	+
811/812	N191F/K368S/M490I	+
813/814	E246V/R291K/V296M/K368S/S444G/M490V	+
815/816	V244R/E246V/K368S/S444G	+
817/818	N191F/G236S/R291K/K368S/M490V	+
819/820	N191F/R291K/K368S/S444G/M490I	+
821/822	V244R/E246V/K368S/M490V	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 730 and are defined as follows: "+" > 3.75, "++" > 8.49, "+++" > 14.16.		

Example 26

Improvements over SEQ ID NO: 730 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0446] SEQ ID NO: 730 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 26.1**.

[0447] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 26.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 50.0; Pre-incubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60.0; Reaction volume (µL): 1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T41mAmUmCmU, T8mAmUmCmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T41mAmUmCmUfC-3'P, T8mAmUmCmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0448] Activity relative to SEQ ID NO: 730 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with

the percent product observed by the reaction with SEQ ID NO: 730 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 26.2**.

Table 26.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 730

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 730)	Pos Control FIOP % product relative to SEQ ID NO: 730
823/824	F262M	+++
825/826	N197M	+++
827/828	K161G	+++
829/830	T173G	++
831/832	M379L	++
833/834	F525W	++
835/836	W382L	++
837/838	E208A	++
839/840	K508D	+
841/842	L343V	+
843/844	H494R	+
845/846	A201E	+
847/848	A416G	+
849/850	K161T	+
851/852	R429S	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 730 and are defined as follows: "+" > 1.09, "++" > 1.26, "+++" > 1.37.		

Example 27

Improvements over SEQ ID NO: 730 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0449] SEQ ID NO: 730 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 27.1**.

[0450] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 27.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 50; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 1; NTP: *mATP-3'P; NTP conc (µM): 600; Oligonucleotide: 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T51fGmUfGmU; Concentration each oligo (µM): 125; Product: 5'-6-FAM-T16mGmUmC*mC*mA-3'P, 5'-6-FAM-T26mCfCmCfG*mA-3'P, 5'-6-FAM-T31mAmAmAfG*mA-3'P, 5'-6-FAM-T51fGmUfGmU*mA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0451] Activity relative to SEQ ID NO: 730 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 730 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 27.2**.

Table 27.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 730		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 730)**	**Pos Control FIOP % product relative to SEQ ID NO: 730**
827/828	K161G	+++
845/846	A201E	+
851/852	R429S	++
853/854	L467M	+++
855/856	L522V	+++
857/858	P337S	+++
859/860	G502M	+++
861/862	A507G	++
863/864	G411E	++
865/866	S164Q	++
867/868	A333N	++
869/870	F525R	++
871/872	L479V/I485V	++
873/874	R484W/I485E	+
875/876	A333S	+
877/878	A463P	+
879/880	E523D	+
881/882	K161Q	+
883/884	H263A	+
885/886	S213G	+
887/888	L522M	+
889/890	D223T	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 730 and are defined as follows: "+" > 1.15, "++" > 1.25, "+++" > 1.38.		

Example 28

Improvements over SEQ ID NO: 742 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0452] SEQ ID NO: 742 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 28.1**.

[0453] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 μM oligonucleotide, 600-1000 μM nucleotide triphosphate, 1-2 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 28.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 63; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (μL): 1; NTP: fCTP-3'P; NTP conc (μM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration each oligo (μM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.0.

[0454] Activity relative to SEQ ID NO: 742 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 742 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 28.2**.

Table 28.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 742		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 742)**	**Pos Control FIOP % product relative to SEQ ID NO: 742**
891/892	K161G/F262M/G411E/A416G	+++
893/894	K161G/N197M/E208G/F262M/A416G	+++
895/896	N197M/E208G/F262M	+++
897/898	K161G/E208G/F262M/G411E/A416G/R429S	+++
899/900	E208G/F262M/R429S	+++
901/902	F262M	+++
903/904	E208G/F262M/A416G/L467M	+++
905/906	E208G/F262M/G411E/A416G	+++
907/908	K161G/F262M/A416G	+++
909/910	K161G/F262M	++
911/912	N197M/F262M	++
913/914	N197M/E208G/L467M	++
915/916	K161G/E208G/L467M	++
917/918	F262M/G411E	++
919/920	F262M/A416G	++
921/922	N197M/E208G/F262M/G411E/R429S/L467M	++

Table 28.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 742		
923/924	K161G/E208G/F262M/G411E/A416G/L467M	++
925/926	E208G/F262M	++
927/928	K161G/F262M/L467M	++
929/930	N197M/E208G/G411E	++
931/932	E208G	++
933/934	N197M/E208G/A416G	+
935/936	N197M/E208G	+
937/938	N197M/E208G/G411E/A416G/L467M	+
939/940	K161G/E208G	+
941/942	K161G/F262M/A416G/L467M	+
943/944	N197M	+
945/946	K161G/F262L/R429S	+
947/948	K161G/F262M/G411E	+
949/950	F262M/A416G/L467M	+
951/952	K161G/F262M/G411E/L467M	+
953/954	E208G/G411E/A416G	+
955/956	E208G/A416G	+
957/958	K161G/R429S	+
959/960	F262M/G411E/A416G	+
961/962	K161G/F262M/G411E/A416G/L467M	+
963/964	F262M/G411E/A416G/R429S	+
965/966	K161G	+
967/968	E208G/A416G/R429S	+
969/970	A416G	+
971/972	G411E/A416G	+
973/974	E208G/G411E/L467M	+
975/976	K161G/A416G/G442R	+
977/978	N197M/F262M/L467M	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 742 and are defined as follows: "+" > 1.61, "++" > 2.81, "+++" > 4.29.		

Example 29

Improvements over SEQ ID NO: 932 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0455] SEQ ID NO: 932 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 29.1**.

[0456] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 29.1 Reaction conditions
Lysis buffer-TEA (50 mM, pH 7.5), 0.1 g/L lysozyme; Reaction buffer-100 mM TEA, 0.6 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%)-25; Pre-incubation Temp (C)-58.5; Pre-incubation Time (min)-60; Reaction temperature (C)-50; Reaction Time (min)-60; Reaction volume (µL) -1; NTP (µM)-fATP-3'P; NTP conc (µM)-480; Oligonucleotide-5'-6-FAM-T15mAmUmCmU, T15mAmUmCmU; Substrate conc (µM)-100 µM FAM-labelled, 400 µM unlabeled; Product (detected)-5'-6-FAM-T15mAmUmCmU(2'dF)A-3'P.

[0457] Activity relative to SEQ ID NO: 932 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 932 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 29.2**.

Table 29.2- Terminal nucleotidyl transferase activity relative to SEQ ID NO: 932		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 932)**	**Pos Control FIOP % product relative to SEQ ID NO: 932**
979/980	L387V	+++
981/982	F262M/H492A	+++
983/984	F262M/L403E	+++
985/986	F262M/Y526E	++
987/988	F262M/T317R	++
989/990	F262M/A339V	++
991/992	F262M/M462L	+
993/994	F262M/Q329L	+
995/996	F262M/L403P	+
997/998	F262M/L403F	+
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 932 and are defined as follows: "+" > 1.75, "++" > 2.15, "+++" > 2.88.		

Example 30

Improvements over SEQ ID NO: 734 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0458] SEQ ID NO: 734 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 30.1**.

[0459] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 µM oligonucleotide, 600-1000 µM nucleotide triphosphate, 1-2 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 30.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 63; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0460] Activity relative to SEQ ID NO: 734 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 734 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 30.2**.

Table 30.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 734		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 734)**	**Pos Control FIOP % product relative to SEQ ID NO: 734**
999/1000	G315K	+++
1001/1002	G315R	+++
1003/1004	E186T	+++
1005/1006	E186L	+++
1007/1008	E186G	+++
1009/1010	W398L	+++
1011/1012	Q353K	+++
1013/1014	L359C	++
1015/1016	E186M	++
1017/1018	D183R	++
1019/1020	Q347S	++
1021/1022	E186V	++
1023/1024	K294G	++
1025/1026	S193R	++
1027/1028	K297S	++
1029/1030	A313S	+
1031/1032	A264L	+
1033/1034	L359A	+
1035/1036	D183S	+
1037/1038	K297A	+
1039/1040	D409P	+
1041/1042	S405P	+
1043/1044	L389V	+
1045/1046	G406R	+
1047/1048	P352A	+
1049/1050	R314S	+
1051/1052	R314A	+
1053/1054	P352N	+
1055/1056	A313H	+

Table 30.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 734
Levels of increased activity were determined pos Control FIOP % product relative to SEQ ID NO: 734 and are defined as follows: "+" > 1.05, "++" > 1.22, "+++" > 1.38.

Example 31

TnT activity with oligonucleotides of 4-7 bp length

Activity improvement of shake-flask purified TnT variants

[0461] TnT variants of SEQ ID NO: 148, 256, and 682 were produced in shake flask and purified as described in Example 3. Variants were evaluated for activity with a series of oligonucleotides with consistent 3' terminal sequences and varying length from 3-7 bp.

[0462] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 1000 µM oligonucleotide, 2000 µM mATP-3'P, 5 µM TnT, 1 µM IPP SEQ ID NO: 1062, 100 mM triethanolamine (pH 7.8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for LCMS analysis as described in Example 33.

Table 31.1
Reaction buffer-100 mM TEA, 1 mM $CoCl_2$, pH 7.8; IPP SEQ ID NO: 1062 (µM)- 1; Reaction temperature (C)- 50; Reaction Time (min)- 180 min; Reaction volume (µL)- 5; TnT concentration (µM)- 1.25 or 5; NTP-mATP-3'P; NTP conc (µM)-2000; Oligonucleotide- mUmCmU, mAmUmCmU, mAmAmUmCmU, mAmAmAmUmCmU, or mAmAmAmAmUmCmU ; Oligonucleotide conc (µM)-2000; Products-mUmCmUmA-3'P, mAmUmCmUmA-3'P, mAmAmUmCmUmA-3'P, mAmAmAmUmCmUmA-3'P, or mAmAmAmAmUmCmUmA-3'P.

[0463] Percent conversion was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area. The results are shown in **Table 31.2**.

Table 31.2					
Reaction substrates			**Percent conversion to product by TnT (see Table 31.1 for list of products detected)**		
oligonucleotide	Nucleotide	uM TnT	SEQ ID NO: 148	SEQ ID NO: 256	SEQ ID NO: 682
mAmAmAmAmUmCmU	mATP-3'P	1.25	++	++	+++
mAmAmAmUmCmU	mATP-3'P	1.25	++	++	++
mAmAmUmCmU	mATP-3'P	1.25	+++	+++	+++
mAmUmCmU	mATP-3'P	1.25	+++	+++	+++
mUmCmU	mATP-3'P	5	+	+	++
Levels of activity were determined by the % conversion to product and defined as follows: "+" from 1-15, "++" from 20-70, "+++" from 71-100.					

Example 32

TnT activity with oligonucleotides of 3-4 bp length

Activity of shake-flask purified TnT variant with short oligonucleotides.

[0464] TnT variant SEQ ID NO: 932 was produced in shake flask and purified as described in Example 3. The variant was evaluated for activity with a series of 4 bp length oligonucleotides with different 3' terminal sequences. Activity with 3 bp oligonucleotides with either a 5' OH or a 5' phosphate were evaluated.

[0465] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 1000 µM oligonucleotide, 2000 µM mATP-3'P, 5 µM TnT, 1 µM IPP SEQ ID NO: 1062, 100 mM triethanolamine (pH 7.8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for LCMS analysis as described in Example 33.

Table 32.1

Reaction buffer-100 mM TEA, 1 mM $CoCl_2$, pH 7.8; IPP SEQ ID NO: 1062 (µM)- 1; Reaction temperature (C)- 50; Reaction Time (min)- 180 min; Reaction volume (µL)- 5; TnT concentration (µM)- 1.25 or 5; NTP-mATP-3'P; NTP conc (µM)-2000; Oligonucleotide- mUmCmU, 5'P-mUmCmU, mAmUmCmU, mC*mA*mGmA, mAfGmUfG, or mGmUmC*mC ; Oligonucleotide conc (µM)-2000; Products-mUmCmUmA-3'P, 5'P-mUmCmUmA-3'P, mAmUmCmUmA-3'P, mC*mA*mGmAmA-3'P, mAfGmUfGmA-3'P, or mGmUmC*mCmA-3'P.

[0466] Percent conversion was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area. The results are shown in **Table 32.2**.

Table 32.2

oligonucleotide	Nucleotide	uM TnT	Percent conversion to product by SEQ ID NO: 932 (nt/aa) (see table 32.1 for list of products detected)
mUmCmU	mATP-3'P	5	+
5'P-mUmCmU	mATP-3'P	5	+++
mAmUmCmU	mATP-3'P	5	+++
mC*mA*mGmA	mATP-3'P	5	+++
mAfGmUfG	mATP-3'P	5	++
mGmUmC*mC	mATP-3'P	5	+++
Levels of activity were determined by the % conversion to product and defined as follows: "+" from 1-30, "++" from 31-90"+++" from 91-100.			

Example 33

HPLC-MS/MS Analysis of Short Oligonucleotides

[0467] The reactions were quenched by the addition on one volume equivalent (5 µL) of a 1 mM EDTA solution. Solutions were mixed well and spun down, then further diluted 10-fold with water. After mixing and

centrifugation, 80 µL of the solution was transferred into a BioRad PCR plate for LCMS analysis. The samples were analyzed according to the LCMS analytical method summarized in **Table 33.1**.

Table 33.1 HPLC-MS/MS Analysis of Short Oligonucleotides

Instrument	Agilent UHPLC 1260 prime series, Agilent 6470B QQQ
Column	Waters Xterra MS C18 50 x 2.1 mm, 5 µm with Phenomenex Security Guard cartridge, C18, 2 mm ID
Mobile Phases	A: 15 mM dibutylamine, 25 mM HFIP (hexafluoro-2-propanol) in water; B: methanol; needle wash: 5% methanol in water.
Gradient	Gradient from 98% A to 30% A and back again:

Time (min)	% A
0.0	98
2.0	98
9.0	55
10.5	30
10.9	30
11.0	98
14.0	98

Flow rate	0.6 mL/min
Run time	14 min
Peak retention times	Some examples *(full list later in table)*: 3-mer (mUmCmU): 1.2 min 4-mer (mAmUmCmU): 4.6 min 5-mer (mAmAmUmCmU): 5.6 min 6-mer (mAmAmAmUmCmU): 6.1 min 7-mer (mAmAmAmAmUmCmU): 6.6 min
Column temperature	50 °C
Injection volume	2-5 µL
MS detection conditions	Ion source: Agilent Jet Stream (AJS) ESI; Scan type: MS2 Scan (400-2050 m/z); polarity: negative; scan time: 500 msec; fragmentor voltage: 200 V; cell accelerator voltage: 3 V; time filter window: 0.03 min
MS source conditions	Drying gas temperature: 275°C; drying gas flow: 8.0 L/min; nebulizer pressure: 35 psi; sheath gas temperature: 350°C; sheath gas flow: 8.0 L/min; capillary voltage: -4000 V; nozzle voltage: -2000
Analyte charge state	$[M-1H]^{1-}$ and/or $[M-2H]^{2-}$

Product Name	Substrate Name	NTP	Reaction	Analyte	m/z Value	Retention Time (min)
3mer +mA +Phos	3-mer (mUmCmU)	mATP-3'P	1	Substrate	896.4	1.2
				Product	1319.6	5.3
5'Phos 3mer +mA +Phos	5'phos-3-mer (5'P-mUmCmU)	mATP-3'P	2	Substrate	976.1	2.3
				Product	1399.4	5.9
				Extra extension	871.0	6.5
4mer +mA +Phos	4-mer (mAmUmCmU)	mATP-3'P	3	Substrate	1239.6	4.6
				Product	830.9	6.2
				Extra extension	1002.5	6.7
4mer +mA +Phos	4-mer (mC*mA*mGmA)	mATP-3'P	4	Substrate	1333.5	5.1
				Product	877.7	6.5

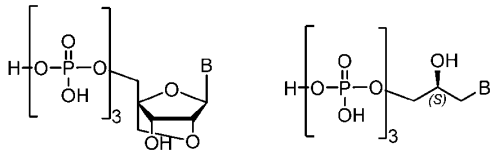
Table 33.1 HPLC-MS/MS Analysis of Short Oligonucleotides						
				Extra extension	1049.6	7.0
4mer +mA +Phos	4-mer (mAfGmUfG)	mATP-3'P	5	Substrate	1294.3	4.7
				Product	1718.0	6.0
				Extra extension	1030.0	6.5
4mer +mA +Phos	4-mer (mGmUmC*mC)	mATP-3'P	6	Substrate	634.7	4.2 & 4.5
				Product	846.2	6.1
				Extra extension	1018.0	6.6
5mer +mA +Phos	5-mer (mAmAmUmCmU)	mATP-3'P	7	Substrate	1583.8	5.6
				Product	1002.6	6.8
				Extra extension	1174.1	7.2
6mer +mA +Phos	6-mer (mAmAmAmUmCmU)	mATP-3'P	8	Substrate	962.5	6.1
				Product	1174.2	7.2
				Extra extension	1346.1	7.5
7mer +mA +Phos	7-mer (mAmAmAmAmUmCmU)	mATP-3'P	9	Substrate	1134.1	6.6
				Product	1345.7	7.5
				Extra extension	1517.8	7.7

Example 34

Activity of SEQ ID NO: 992 in the extension of oligonucleotide acceptor molecules with glycol nucleic acid (GNA) and locked nucleic acid (LNA) nucleotide triphosphate donors

Screening of shake-flask purified TnT variant with (*S*)-GNA and LNA NTP donors.

[0468] TnT variant SEQ ID NO: 992 was produced in shake flask and purified as described in Example 3.



Scheme 3. Structure of LNA-NTP and (S)-GNA-ATP screened with **SEQ ID NO: 992.**

[0469] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 100 µM oligonucleotide, 500 µM nucleotide triphosphate, 5 µM TnT, 1 µM IPP SEQ ID NO: 1062, 100 mM triethanolamine (pH 7.8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature

and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 34.1
Reaction buffer-100 mM TEA, 1 mM $CoCl_2$, pH 7.8; IPP SEQ ID NO: 1062 (μM)- 1; Reaction temperature (C)- 50; Reaction Time (min)- 60 min; Reaction volume (μL)- 1; TnT concentration (μM)- 5; NTP- (*S*)-GNA-ATP, +ATP, +GTP, +CTP, +UTP; NTP conc (μM)-500; Oligonucleotide- 5'-6-FAM-T9mAmAmAmUmCmU; Oligonucleotide conc (μM)-100 (labeled) ; Products- 5'-6-FAM-T9mAmAmAmUmCmU(sgna)A, 5'-6-FAM-T9mAmAmAmUmCmU+A, 5'-6-FAM-T9mAmAmAmUmCmU+A+A, 5'-6-FAM-T9mAmAmAmUmCmU+G, 5'-6-FAM-T9mAmAmAmUmCmU+G+G, 5'-6-FAM-T9mAmAmAmUmCmU+C, 5'-6-FAM-T9mAmAmAmUmCmU+U, 5'-6-FAM-T9mAmAmAmUmCmU+U+U.

[0470] Activity of SEQ ID NO: 992 was calculated as the percent product defined as the sum of the area of products, either the single base or double base extension product, divided by the sum of the total peak area. The results are shown in **Table 34.2**.

Table 34.2-Activity of SEQ ID NO: 992 with (*S*)-GNA-ATP and LNA-NTP donors		
	% Single-base extension product	% Two-base extension product
(*S*)-GNA-ATP	+++	~
+ATP	+++	++
+GTP	+++	+
+CTP	+++	~
+UTP	+++	+
Percent conversion to single and double base extension products by SEQ ID NO: 992 are defined as follows: "~" 0, "+" 1-10, "++" 10-75, "+++" > 75.		

Example 35

Improvements over SEQ ID NO: 932 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0471] SEQ ID NO: 932 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 35.1**.

[0472] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 500-600 μM oligonucleotide, 600-1000 μM nucleotide triphosphate, 1-2 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 35.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Pre-incubation Temp (°C): 63; Pre-incubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 2000; Oligonucleotide: 5'-6-FAM-T46mUmCmUmU, T8mUmCmUmU; Concentration each oligo (µM): 125 (FAM label), 875 (no label); Product: 5'-6-FAM-T46mUmCmUmUfC-3'P, T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0473] Activity relative to SEQ ID NO: 932 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 932 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 35.2**.

Table 35.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 932		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 932)**	**Pos Control FIOP % product relative to SEQ ID NO: 932**
1129/1130	F262M/L387T	+
1131/1132	F262M/I340V	+
1133/1134	F262M/E371R	+
1135/1136	F262M/K377R	+
1137/1138	F262M/E371G/E443D	+
1139/1140	S184K/F262M	+
1141/1142	F262M/P363G	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 932 and are defined as follows: "+" > 1.6.		

Example 36

Improvements over SEQ ID NO: 992 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0474] SEQ ID NO: 992 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 36.1**.

[0475] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 36.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 10; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 120.0; Reaction volume (µL): 0.1; NTP: *mGTP-3'P; NTP conc (µM): 600.0; Labeled oligonucleotide: 5'-6-FAM-T16mAfGmAfG, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T51mAmUmCfC; Concentration each labeled oligo (µM): 125.0; Labeled products: 5'-6-FAM-T16mAfGmAfG*mG-3'P, 5'-6-FAM-T26mCfCmCfG*mG-3'P, 5'-6-FAM-T31mAmAmAfG*mG-3'P, 5'-6-FAM-T51mAmUmCfC*mG-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0476] Activity relative to SEQ ID NO: 992 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 992 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 36.2**.

Table 36.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 992		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 992)**	**FIOP activity relative to SEQ ID NO: 992**
1143/1144	R60H/S193T/A264L/K297S	++
1145/1146	G208E/R273M/T295N	++
1147/1148	G208E/R273L/K294T/P407G	++
1149/1150	R273L	++
1151/1152	K297S	++
1153/1154	R60H/H263A/A264L/K297S/T300S/F525W	+
1155/1156	S193T/K297A	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 992 and are defined as follows: "+" > 1.1, "++" > 1.3.		

Example 37

Improvements over SEQ ID NO: 1144 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0477] SEQ ID NO: 1144 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 37.1**.

[0478] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 37.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 66.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60.0; Reaction volume (µL): 1.0; NTP fCTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 125; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 875; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0479] Activity relative to SEQ ID NO: 1144 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1144 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 37.2**.

Table 37.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1144		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1144)**	**FIOP activity relative to SEQ ID NO: 1144**
1157/1158	Q260R/L264A	+++
1159/1160	L264A	+++
1161/1162	S231T	+++
1163/1164	M238F	+++
1165/1166	Q251G	+++
1167/1168	A252S	+++
1169/1170	E233I	++
1171/1172	Q251S	++
1173/1174	S231M	++
1175/1176	Q251W	++
1177/1178	I237L	++
1179/1180	M238L	++
1181/1182	M238V	++
1183/1184	I230C	+
1185/1186	I230L	+
1187/1188	Q251R	+
1189/1190	S231C	+
1191/1192	A252M	+
1193/1194	Q251E	+
1195/1196	V250G	+
1197/1198	I241L	+
1199/1200	Q251M	+
1201/1202	V234T	+
1203/1204	W229F	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1144 and are defined as follows: "+" > 1.3, "++" > 1.6, "+++" > 1.9.		

Example 38

Improvements over SEQ ID NO: 1144 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0480] SEQ ID NO: 1144 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 38.1**.

[0481] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 38.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 66.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60.0; Reaction volume (µL): 1.0; NTP fCTP-3'P; NTP conc (µM): 600.0; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 125; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 875; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0482] Activity relative to SEQ ID NO: 1144 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1144 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 38.2**.

Table 38.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1144

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1144)	FIOP activity relative to SEQ ID NO: 1144
1205/1206	Q329L/A339V/I340V/L403E	+++
1207/1208	Q329L/A339V/L403E/E443D	+++
1209/1210	Q329L/A339V/I340V/L403E/Y526E	+++
1211/1212	A339V	+++
1213/1214	Q329L/Y526E	++
1215/1216	A339V/L403E	++
1217/1218	A339V/I340V/L387V/Y526E	++
1219/1220	Q329L	++
1221/1222	Q329L/A339V/I340V	++
1223/1224	A339V/Y526E	+
1225/1226	A339V/I340V/V428L	+
1227/1228	E443D/Y526E	+

Table 38.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1144		
1229/1230	E371G/L403E/Y526E	+
1231/1232	Q329L/A339V/E371G/L387T	+
1233/1234	Q329L/A339V/I340V/E371G/L387T	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1144 and are defined as follows: "+" > 1.4, "++" > 4.2, "+++" > 6.6.		

Example 39

Improvements over SEQ ID NO: 1212 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0483] SEQ ID NO: 1212 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 39.1**.

[0484] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 39.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (μL): 1.0; NTP: *mATP-3'P; NTP conc (μM): 1000; Labeled oligonucleotide: 5'-6-FAM-T26fCfAfUmG , 5'-6-FAM-T21mUmAmG*mA , 5'-6-FAM-T31mUfCmAmU , 5'-6-FAM-T51fGmUfGmU ; Concentration each labeled oligo (μM): 125.0; Labeled products: 5'-6-FAM-T26fCfAfUmG*mA-3'P, 5'-6-FAM-T21mUmAmG*mA*mA-3'P, 5'-6-FAM-T31mUfCmAmU*mA-3'P, 5'-6-FAM-T51fGmUfGmU*mA-3'P;; IPP: SEQ ID NO: 1062; IPP (μM): 1.0.

[0485] Activity relative to SEQ ID NO: 1212 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1212 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 39.2**.

Table 39.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1212		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1212)**	**FIOP activity relative to SEQ ID NO: 1212**
1235/1236	Q329L	++
1237/1238	I340V/G460V	++
1239/1240	Q376S	+
1241/1242	H492A	+
1243/1244	S184K/Q329L/L403E/S404G/H492A	+
1245/1246	S184K/L403F/S404G/Y526E	+
1247/1248	I340M	+
1249/1250	Q329L/Y526E	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1212 and are defined as follows: "+" > 1.0, "++" > 1.2.		

Example 40

Improvements over SEQ ID NO: 1236 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0486] SEQ ID NO: 1236 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 40.1**.

[0487] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 40.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 3.13; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mUTP-3'P; NTP conc (µM): 1200; Labeled oligonucleotide: 5'-6-FAM-T10AmC*mA*mG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56fGmUfCmU; Concentration each labeled oligo (µM): 100; Unlabeled oligonucleotide: T8AmC*mA*mG, T8mCfCmGfG, T8fAmUmUfG, T8fGmUfCmU; Concentration each unlabeled oligo (µM): 125.0; Labeled products: 5'-6-FAM-T10AmC*mA*mGmU-3'P, 5'-6-FAM-T36mCfCmGfGmU-3'P, 5'-6-FAM-T46fAmUmUfGmU-3'P, 5'-6-FAM-T56fGmUfCmUmU-3'P; Unlabeled products: T8AmC*mA*mGmU-3'P, T8mCfCmGfGmU-3'P, T8AmUmUfGmU-3'P, T8fGmUfCmUmU-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 0.2.

[0488] Activity relative to SEQ ID NO: 1236 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1236 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 40.2**.

Table 40.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1236

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1236)	FIOP activity relative to SEQ ID NO: 1236
1251/1252	I450V	++
1253/1254	W325Y/L329Q	++
1255/1256	R190A	++
1257/1258	T319Q	++
1259/1260	W325S/L329Q	++
1261/1262	D322E/L329Q	++
1263/1264	R190T	++
1265/1266	A415G	+
1267/1268	F525R	+
1269/1270	R190L	+
1271/1272	F525I	+
1273/1274	R484A	+
1275/1276	L343T	+
1277/1278	M379L	+
1279/1280	D198E	+
1281/1282	A463S	+
1283/1284	L202M	+
1285/1286	L329Q	+
1287/1288	H263W	+
1289/1290	H263S	+
1291/1292	E523D	+
1293/1294	R480M	+
1295/1296	L479V	+
1297/1298	R190V	+
1299/1300	K212R	+
1301/1302	S297P	+
1303/1304	Q477T	+
1305/1306	L174W	+
1307/1308	S297G	+
1309/1310	R318Q	+
1311/1312	F525V	+
1313/1314	D412Q	+
1315/1316	R456M	+
1317/1318	L343R	+
1319/1320	R190F	+
1321/1322	W325G/L329Q	+
1323/1324	L419A	+

Table 40.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1236		
1325/1326	L343V	+
1327/1328	H263A	+
1329/1330	K448R	+
1331/1332	L202S	+
1333/1334	A201D	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1236 and are defined as follows: "+" > 1.03, "++" > 1.13.		

Example 41

Improvements over SEQ ID NO: 1236 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0489] SEQ ID NO: 1236 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 41.1**.

[0490] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 41.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 70; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60.0; Reaction volume (µL): 1.0; NTP fUTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 975; Labeled product: 5'-6-FAM-T46mUmCmUmUfU-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0491] Activity relative to SEQ ID NO: 1236 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1236 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 41.2**.

Table 41.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1236		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1236)**	**FIOP activity relative to SEQ ID NO: 1236**
1335/1336	E203M	+++
1337/1338	D199F	+++
1339/1340	L389V	+++
1341/1342	P352E	+++
1343/1344	T300N	+++
1345/1346	A313R	+++
1347/1348	P407L	+++
1349/1350	P407V	+++
1351/1352	S354G	+++
1353/1354	Q281G	++
1355/1356	T295A	++
1357/1358	G196H	++
1359/1360	Y280W	++
1361/1362	S404V	++
1363/1364	F194L	++
1365/1366	Q281H	++
1367/1368	D409E	++
1369/1370	Q179L	++
1371/1372	G406N	+
1373/1374	P352L	+
1375/1376	L264R	+
1377/1378	Q353V	+
1379/1380	Q281C	+
1381/1382	T300L	+
1383/1384	R314T	+
1385/1386	N175V	+
1387/1388	D183V	+
1389/1390	Q353L	+
1391/1392	S404L	+
1393/1394	Q281S	+
1395/1396	Q281L	+
1397/1398	G406T	+
1399/1400	A313H	+
1401/1402	S404E	+
1403/1404	Y192A	+
1405/1406	S404Q	+
1407/1408	A408L	+
1409/1410	G208Q	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1236 and are defined as follows: "+" > 1.2, "++" > 1.4, "+++" > 1.6.		

Example 42

Improvements over SEQ ID NO: 1264 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0492] SEQ ID NO: 1264 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 42.1**.

[0493] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 42.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 1.0; NTP: mATP-3'P; NTP conc (µM): 6000; Labeled oligonucleotide: 5'-6-FAM-T36mCfCmGfG , 5'-6-FAM-T41mAfUfCfC , 5'-6-FAM-T51mU*fA*fAfG , 5'-6-FAM-T56mA*fC*mAfA ; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mCfCmGfG, T8mAfUfCfC, T8mU*fA*fAfG, T8mA*fC*mAfA; Concentration each unlabeled oligo (µM): 475; Labeled products: 5'-6-FAM-T36mCfCmGfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P, 5'-6-FAM-T51mU*fA*fAfGmA-3'P, 5'-6-FAM-T56mA*fC*mAfAmA-3'P; Unlabeled products: T8mCfCmGfGmA-3'P, T8mAfUfCfCmA-3'P, T8mU*fA*fAfGmA-3'P, T8mA*fC*mAfAmA-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0494] Activity relative to SEQ ID NO: 1264 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1264 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 42.2**.

Table 42.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1264		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1264)**	**FIOP activity relative to SEQ ID NO: 1264**
1411/1412	M379L/I420V	+++
1413/1414	A313R/M379L/F525V	+++
1415/1416	T295A/M379L/I420V	+++
1417/1418	I420V	++

Table 42.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1264		
1419/1420	T300S/M379L/I420V	++
1421/1422	T295A/I420V	++
1423/1424	L264S/I420V/F525V	++
1425/1426	E342S/I420V	++
1427/1428	H263S/T295A/T300S/I420V	++
1429/1430	T190G/T300S/F525V	++
1431/1432	T300S/I420V	+
1433/1434	A313R	+
1435/1436	T190G/H263S/T295A/T300S/E342S/V393T	+
1437/1438	L264S/T300S/I420V	+
1439/1440	L264S/M379L	+
1441/1442	F525V	+
1443/1444	E342S	+
1445/1446	H263S/T295A/I420V	+
1447/1448	L264S/I420V	+
1449/1450	M379L	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1264 and are defined as follows: "+" > 1.15, "++" > 1.24, "+++" > 1.41.		

Example 43

Improvements over SEQ ID NO: 1264 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0495] SEQ ID NO: 1264 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 43.1**.

[0496] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 43.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 6000; Labeled oligonucleotide: 5'-6-FAM-T16*fA*mAmGfAfG, 5'-6-FAM-T25mAmGfUfGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8*fA*mAmGfAfG, T8mAmGfUfGfG; Concentration each unlabeled oligo (µM): 975; Labeled products: 5'-6-FAM-T16*fA*mAmGfAfGfC-3'P, 5'-6-FAM-T25mAmGfUfGfGfC-3'P; Unlabeled products: T8*fA*mAmGfAfGfC-3'P, T8mAmGfUfGfGfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.0.

[0497] Activity relative to SEQ ID NO: 1264 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1264 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 43.2.**

Table 43.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1264		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1264)**	**FIOP activity relative to SEQ ID NO: 1264**
1451/1452	K392Q	++
1453/1454	V393T	++
1455/1456	F418C	++
1457/1458	T190M	+
1459/1460	L174V	+
1461/1462	Q353V	+
1463/1464	T190R/F194M	+
1465/1466	W395E	+
1467/1468	P407Q	+
1469/1470	Q260R	+
1471/1472	Q353L	+
1473/1474	W395T	+
1475/1476	G411K	+
1477/1478	A408N	+
1479/1480	T190R/T193I	+
1481/1482	W395M	+
1483/1484	S405Q	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1264 and are defined as follows: "+" > 1.02, "++" > 1.05.		

Example 44

Improvements over SEQ ID NO: 1264 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0498] SEQ ID NO: 1264 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 44.1**.

[0499] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 44.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 69; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP fUTP-3'P; NTP conc (μM): 2000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (μM): 975; Labeled product: 5'-6-FAM-T46mUmCmUmUfU-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (μM): 1.0.

[0500] Activity relative to SEQ ID NO: 1264 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1264 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 44.2**.

Table 44.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1264		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1264)**	**FIOP activity relative to SEQ ID NO: 1264**
1463/1464	T190R/F194M	++
1471/1472	Q353L	+
1483/1484	S405Q	+
1485/1486	T190R/T193S	++
1487/1488	T190R	++
1489/1490	S405V	++
1491/1492	P407Y	++
1493/1494	A408V	+
1495/1496	D409V	+
1497/1498	T190R/T193V	+
1499/1500	P407G	+
1501/1502	T190R/T193G	+

Table 44.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1264
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1264 and are defined as follows: "+" > 1.2, "++" > 1.3.

Example 45

Improvements over SEQ ID NO: 1414 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0501] SEQ ID NO: 1414 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 45.1**.

[0502] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 45.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 1.0; NTP: mATP-3'P; NTP conc (µM): 6000; Labeled oligonucleotide: 5'-6-FAM-T16*fC*mAfAfA, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T25mAmGfUfGfG, 5'-6-FAM-T41mAfUfCfC ; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8*fC*mAfAfA, T8mAmUfUmGfG, T8mAmGfUfGfG, T8mAfUfCfC; Concentration each unlabeled oligo (µM): 475; Labeled products: 5'-6-FAM-T16*fC*mAfAfAmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T25mAmGfUfGfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P; Unlabeled products: T8*fC*mAfAfAmA-3'P, T8mAmUfUmGfGmA-3'P, T8mAmGfUfGfGmA-3'P, T8mAfUfCfCmA-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0503] Activity relative to SEQ ID NO: 1414 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1414 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 45.2**.

Table 45.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1414		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1414)**	**FIOP activity relative to SEQ ID NO: 1414**
1503/1504	E203M/G208Q/T295D/Y308H/D409E	++
1505/1506	E203M/G208Q/T295A/Y308H/D409E	++
1507/1508	F194L	++
1509/1510	T295A/Y308H/D409E	+
1511/1512	H263S/T300N/Q353V	+
1513/1514	T300N	+
1515/1516	E203M/G208Q/T295D/L389V/D409E	+
1517/1518	Q169L/D409E	+
1519/1520	P352A/Q353V	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1414 and are defined as follows: "+" > 1.13, "++" > 1.21.		

Example 46

Improvements over SEQ ID NO: 1504 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0504] SEQ ID NO: 1504 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 46.1**.

[0505] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 46.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 6000; Labeled oligonucleotide: 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T25mAmGfUfGfG, 5'-6-FAM-T35fAfGfUmGfG, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mGfUfAfC, T8mAmGfUfGfG, T8fAfGfUmGfG, T8mGfUmGfG; Concentration each unlabeled oligo (µM): 475; Labeled products: 5'-6-FAM-T21mGfUfAfCfC-3'P, 5'-6-FAM-T25mAmGfUfGfGfC-3'P, 5'-6-FAM-T35fAfGfUmGfGfC-3'P, 5'-6-FAM-T41mGfUmGfGfC-3'P; Unlabeled products: T8mGfUfAfCfC-3'P, T8mAmGfUfGfGfC-3'P, T8fAfGfUmGfGfC-3'P, T8mGfUmGfGfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0506] Activity relative to SEQ ID NO: 1504 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1504 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 46.2**.

Table 46.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1504

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1504)	FIOP activity relative to SEQ ID NO: 1504
1521/1522	L174V/Q260R/H263S/Q353L/S405Q/A408V	++
1523/1524	Q353L/I420V	++
1525/1526	I420V	++
1527/1528	Q260R/Q353V/I420V	+
1529/1530	P407S/E409D/I420V	+
1531/1532	L174V/S405Q/P407Y/I420V	+
1533/1534	P407Y/A408V/I420V	+
1535/1536	L174V/Q353V	+
1537/1538	H263S/P352A/Q353V/S405Q/A408V/I420V	+
1539/1540	H263S/Q353V/I420V	+
1541/1542	L174V/P407Y/E409D/I420V	+
1543/1544	Q353V/I420V	+
1545/1546	H263S/I420V	+
1547/1548	S405Q/A408V/E409D/I420V	+
1549/1550	Q260R/Q353V	+

Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1504 and are defined as follows: "+" > 1.1, "++" > 1.2.

Example 47

Improvements over SEQ ID NO: 1504 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0507] SEQ ID NO: 1504 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 47.1**.

[0508] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 47.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 68.5; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP fUTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 975; Labeled product: 5'-6-FAM-T41mAmUmCmUfU-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0509] Activity relative to SEQ ID NO: 1504 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1504 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 47.2**.

Table 47.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1504		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1504)**	**FIOP activity relative to SEQ ID NO: 1504**
1551/1552	L389V	+++
1553/1554	D295T/S297P	+++
1555/1556	D295T/W301V/H308Y	+++
1557/1558	H308Y/R314I	+++
1559/1560	N197R/M203E	++
1561/1562	H263R	++
1563/1564	H308Y	++
1565/1566	C390L	++
1567/1568	C390M	++
1569/1570	S461A	++
1571/1572	Y394N	++
1573/1574	S405V/E409D	++
1575/1576	D295T/S297G	++
1577/1578	Q281G	++
1579/1580	H308L	++
1581/1582	V328A	++
1583/1584	S261R	++
1585/1586	H263K	++
1587/1588	S261V	++
1589/1590	S404M/E409D	+
1591/1592	Y394S	+
1593/1594	R484W	+
1595/1596	Q281S/N440D	+
1597/1598	S261G	+
1599/1600	K133E/N197Q/M203E	+

Table 47.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1504		
1601/1602	S404I/E409D	+
1603/1604	S405A/E409D	+
1605/1606	D322E	+
1607/1608	Y192H	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1504 and are defined as follows: "+" > 1.5, "++" > 1.9, "+++" > 2.4.		

Example 48

Improvements over SEQ ID NO: 1522 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0510] SEQ ID NO: 1522 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 48.1**.

[0511] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 48.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 48; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (μL): 1.0; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T51 mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mCfCmGfG, T8mGfUmGfG, T8mU*fA*fAfG, T8mA*fC*mAfA; Concentration each unlabeled oligo (μM): 2360; Labeled products: 5'-6-FAM-T36mCfCmGfGmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P, 5'-6-FAM-T51mU*fA*fAfGmA-3'P, 5'-6-FAM-T56mA*fC*mAfAmA-3'P; Unlabeled products: T8mCfCmGfGmA-3'P, T8mGfUmGfGmA-3'P, T8mU*fA*fAfGmA-3'P, T8mA*fC*mAfAmA-3'P; IPP: SEQ ID NO: 1062 ; IPP (μM): 1.0.

[0512] Activity relative to SEQ ID NO: 1522 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1522 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 48.2**.

Table 48.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1522		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1522)**	**FIOP activity relative to SEQ ID NO: 1522**
1609/1610	E52G	++
1611/1612	T193G	++
1613/1614	T182V	++
1615/1616	N175S	++
1617/1618	K195T	++
1619/1620	N175E	++
1621/1622	G196V	+
1623/1624	K278R	+
1625/1626	T193A	+
1627/1628	K195R	+
1629/1630	A201S	+
1631/1632	T193V	+
1633/1634	N175T	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1522 and are defined as follows: "+" > 1.02, "++" > 1.05.		

Example 49

Improvements over SEQ ID NO: 1522 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0513] SEQ ID NO: 1522 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 49.1**.

[0514] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 49.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 69; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 60.0; Reaction volume (µL): 0.1; NTP fCTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 975; Labeled product: 5'-6-FAM-TT41mAmUmCmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0515] Activity relative to SEQ ID NO: 1522 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1522 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 49.2**.

Table 49.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1522		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1522)**	**FIOP activity relative to SEQ ID NO: 1522**
1623/1624	K278R	++
1635/1636	S297W	+++
1637/1638	L479M	+++
1639/1640	T193R	+++
1641/1642	K195W	+++
1643/1644	F418T	++
1645/1646	K195F	++
1647/1648	L479T	++
1649/1650	S297V	++
1651/1652	S299G	++
1653/1654	H494L	++
1655/1656	S299H	++
1657/1658	F418S	++
1659/1660	H494Q	++
1661/1662	W301A	++
1663/1664	S297A	+
1665/1666	D412V	+
1667/1668	D412S	+
1669/1670	S299P	+
1671/1672	T275R	+
1673/1674	H494V	+
1675/1676	A201P	+
1677/1678	T275S	+
1679/1680	H494M	+
1681/1682	G196R	+
1683/1684	M282N	+
1685/1686	L462F	+
1687/1688	A201R	+

Table 49.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1522		
1689/1690	S297P	+
1691/1692	G272R	+
1693/1694	T173P	+
1695/1696	S461V	+
1697/1698	R314A	+
1699/1700	S297T	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1522 and are defined as follows: "+" > 1.1, "++" > 1.5, "+++" > 2.0.		

Example 50

Improvements over SEQ ID NO: 1522 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0516] SEQ ID NO: 1522 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 50.1**.

[0517] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 50.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mGTP-3'P; NTP conc (µM): 6000; Labeled oligonucleotide: 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T35fAfGfUmGfG, 5'-6-FAM-T41mAfUfCfC ; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8mAmGfAmGfG, T8fAfGfUmGfG, T8mAfUfCfC; Concentration each unlabeled oligo (µM): 475; Labeled products: 5'-6-FAM-T11fUfUfUfCmG-3'P, 5'-6-FAM-T20mAmGfAmGfGmG-3'P, 5'-6-FAM-T35fAfGfUmGfGmG-3'P, 5'-6-FAM-T41mAfUfCfCmG-3'P; Unlabeled products: T8fUfUfUfCmG-3'P, T8mAmGfAmGfGmG-3'P, T8fAfGfUmGfGmG-3'P, T8mAfUfCfCmG-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0518] Activity relative to SEQ ID NO: 1522 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with

the percent product observed by the reaction with SEQ ID NO: 1522 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 50.2**.

Table 50.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1522		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1522)**	**FIOP activity relative to SEQ ID NO: 1522**
1701/1702	F194L/N197M/T319V/I361V/I420V	+++
1703/1704	F194L/T319V/I361V/I420V	+++
1705/1706	F194L/T319V/I420V	++
1707/1708	V174L/F194L/N197M/R260Q/S263H/T319V/L353V/E409D /I420V	++
1709/1710	V174L/F194L/K195Q/N197M/T319V/I361V/I420V	++
1711/1712	F194L/K195Q/T319V/I420V	++
1713/1714	F194L/K195Q/N197M/T319V	++
1715/1716	V174L/F194L/N197M/R260Q/S263H/L353V/E409D/I420V	++
1717/1718	W325V/G406D	++
1719/1720	F194L/K195Q/I361V	++
1721/1722	F194L/T319V	+
1723/1724	K195Q/T319V/I361V/I420V	+
1725/1726	T319V/I420V	+
1727/1728	I361V/I420V	+
1729/1730	F194L/N197M	+
1731/1732	T300N/G406D/E409D	+
1733/1734	T300N/L353V/I420V/V525T	+
1735/1736	T300N/W325V/G406D	+
1737/1738	N197M/I420V	+
1739/1740	K195Q/T319V/I420V	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1522 and are defined as follows: "+" > 1.2, "++" > 1.4, "+++" > 1.7.		

Example 51

Improvements over SEQ ID NO: 1702 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0519] SEQ ID NO: 1702 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 51.1**.

[0520] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a

peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 51.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 12.5; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8*fU*fUfAfU, T8mAmUfUmGfG, T8mGfUmGfG; Concentration each unlabeled oligo (µM): 2360; Labeled products: 5'-6-FAM-T11fUfUfUfCmA-3'P, 5'-6-FAM-T16*fU*fUfAfUmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P; Unlabeled products: T8fUfUfUfCmA-3'P, T8*fU*fUfAfUmA-3'P, T8mAmUfUmGfGmA-3'P, T8mGfUmGfGmA-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0521] Activity relative to SEQ ID NO: 1702 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1702 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 51.2**.

Table 51.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1702		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1702)**	**FIOP activity relative to SEQ ID NO: 1702**
1741/1742	S261V/E409D	+
1743/1744	S297P	+
1745/1746	E409D	+
1747/1748	T300N	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1702 and are defined as follows: "+" > 1.0.		

Example 52

Improvements over SEQ ID NO: 1742 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0522] SEQ ID NO: 1742 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 52.1**.

[0523] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and

1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 52.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 70; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP fCTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU ; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0524] Activity relative to SEQ ID NO: 1742 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1742 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 52.2**.

Table 52.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1742		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1742)**	**FIOP activity relative to SEQ ID NO: 1742**
1749/1750	S297V/T300N	++
1751/1752	S297A/T300N	++
1753/1754	T193G/S297V/T300N/R314A/G442R	++
1755/1756	N175S/S297V/T300N/L462F	++
1757/1758	N175S/S297V/T300N	++
1759/1760	N175S/T300N/R314A	++
1761/1762	K195T/S297A/T300N/L462F	++
1763/1764	K195T/S297V/T300N/L462F	++
1765/1766	N175S/T300N	++
1767/1768	N175S/T193G/S297V/T300N	++
1769/1770	T193G/K195T/S297V/T300N/R314A	+
1771/1772	E52G/A201S/C390M	+
1773/1774	N175S/T300N/G442R	+
1775/1776	L221Q/S297A/T300N	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1742 and are defined as follows: "+" > 1.2, "++" > 1.3.		

Example 53

Improvements over SEQ ID NO: 1742 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0525] SEQ ID NO: 1742 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 53.1**.

[0526] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 53.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50.0; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90.0; Reaction volume (µL): 1.0; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T36mCfCmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8*fU*fUfAfU, T8mAmUfUmGfG, T8mCfCmGfG; Concentration each unlabeled oligo (µM): 2360; Labeled products: 5'-6-FAM-T11fUfUfUfCmA-3'P, 5'-6-FAM-T16*fU*fUfAfUmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P; Unlabeled products: T8fUfUfUfCmA-3'P, T8*fU*fUfAfUmA-3'P, T8mAmUfUmGfGmA-3'P, T8mCfCmGfGmA-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0527] Activity relative to SEQ ID NO: 1742 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1742 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 53.2**.

Table 53.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1742		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1742)**	**FIOP activity relative to SEQ ID NO: 1742**
1777/1778	K303R	++
1779/1780	S404E/D409E	++
1781/1782	K397S	++
1783/1784	G406P/D409E	++
1785/1786	S404G/D409E	++
1787/1788	G406A/D409E	++
1789/1790	G196L	++

Table 53.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1742		
1791/1792	V319P	++
1793/1794	G196D	+
1795/1796	R152H/Q281S	+
1797/1798	S297T	+
1799/1800	M197I	+
1801/1802	Q477S	+
1803/1804	T182V	+
1805/1806	M474L	+
1807/1808	V261T	+
1809/1810	G196S	+
1811/1812	T307N	+
1813/1814	Q281S	+
1815/1816	Q405S/D409E	+
1817/1818	D409E	+
1819/1820	A463P	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1742 and are defined as follows: "+" > 1.05, "++" > 1.12.		

Example 54

Improvements over SEQ ID NO: 1742 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0528] SEQ ID NO: 1742 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 54.1**.

[0529] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 54.1 Reaction conditions
Reaction buffer 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 72; Preincubation Time (min): 60.0; Reaction temperature (°C): 50.0; Reaction Time (min): 90.0; Reaction volume (µL): 0.1; NTP fCTP-3'P; NTP conc (µM): 2000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062 ; IPP (µM): 1.0.

[0530] Activity relative to SEQ ID NO: 1742 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1742 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 54.2**.

Table 54.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1742		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1742)**	**FIOP activity relative to SEQ ID NO: 1742**
1777/1778	K303R	++
1779/1780	S404E/D409E	++
1783/1784	G406P/D409E	+++
1787/1788	G406A/D409E	+
1791/1792	V319P	+
1795/1796	R152H/Q281S	++
1797/1798	S297T	+
1799/1800	M197I	+
1813/1814	Q281S	++
1821/1822	L479M	+++
1823/1824	Q477E	+++
1825/1826	P274S	+++
1827/1828	Q281G	+++
1829/1830	T190R	+++
1831/1832	H492G	++
1833/1834	H308Y	++
1835/1836	S404M/D409E	++
1837/1838	Q281H	++
1839/1840	L360V	++
1841/1842	Q208E	++
1843/1844	Q281A	++
1845/1846	S461A	++
1847/1848	L479T	++
1849/1850	Q208T	++
1851/1852	S404V/D409E	+
1853/1854	K303T	+
1855/1856	G406M/D409E	+
1857/1858	A321T	+

Table 54.2-Terminal nucleotidyl transferase activity relative to SEQ ID NO: 1742		
1859/1860	V319L	+
1861/1862	T307D	+
1863/1864	C417G	+
1865/1866	S299N	+
1867/1868	T307E	+
1869/1870	T307M	+
1871/1872	V319G	+
1873/1874	V319M	+
1875/1876	G495A	+
1877/1878	R313A	+
1879/1880	G460D	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1742 and are defined as follows: "+" > 1.5, "++" > 2.5, "+++" > 5.0.		

Example 55

HPLC-MS/MS Analysis of Short Oligonucleotides

Method for evaluating reactions with short oligonucleotides

[0531] The reactions were quenched by the addition on one volume equivalent (5 µL) of a 1 mM EDTA solution. Solutions were mixed well and spun down, then further diluted 10-fold with water. After mixing and centrifugation, 80 µL of the solution was transferred into a BioRad PCR plate for LCMS analysis. The samples were analyzed according to the LCMS analytical methods summarized in Tables 55.1, 55.2, and 55.3.

Table 55.1 HPLC-MS/MS Analysis of Short Oligonucleotides (2-6mers)	
Instrument	Agilent UHPLC 1260 prime series, Agilent 6470B QQQ
Column	Waters Xterra MS C18 150 x 2.1 mm, 5 µm with Phenomenex Security Guard cartridge, C18, 2 mm ID
Mobile phases	A: 15 mM dibutylamine, 25 mM HFIP (hexafluoro-2-propanol) in water; B: methanol; needle wash: 20% methanol in water.
Gradient	Gradient from 90% A to 10% A and back again: **Time (min)** / **% A** 0.0 / 90 0.2 / 90 7.5 / 50 8.0 / 10 8.4 / 10 8.5 / 90 13.0 / 90
Run time	13.0 min
Flow rate	0.4 mL/min
Peak retention times	Samples typically elute between 4-7 minutes.

Table 55.1 HPLC-MS/MS Analysis of Short Oligonucleotides (2-6mers)						
		Note: retention times can shift depending on preparation and age of mobile phase A.				
Column temperature		50 °C				
Injection volume		2-5 µL				
MS detection conditions		Ion source: Agilent Jet Stream (AJS) ESI; Scan type: MS2 Scan (550-2050 m/z); polarity: negative; scan time: 500 msec; fragmentor voltage: 200 V; cell accelerator voltage: 3 V; time filter window: 0.03 min				
MS source conditions		Drying gas temperature: 275°C; drying gas flow: 12.0 L/min; nebulizer pressure: 35 psi; sheath gas temperature: 350°C; sheath gas flow: 12.0 L/min; capillary voltage: -4000 V; nozzle voltage: -2000				
Analyte charge state		$[M-1H]^{1-}$ and/or $[M-2H]^{2-}$				
Product Name	**Substrate Name**	**NQP**		**Analyte**	**m/z Value**	**Retention Time (min)**
mC*mU*mA +mG +Phos	mC*mU*mA	mGQP		Substrate	951.2	4.4
				Product	1390.3	5.5
mU*fU*mA +mG +Phos	mU*fU*mA	mGQP		Substrate	940.1	4.6
				Product	1379.3	5.7
(VPU)*fU*mAmG +mG +Phos	(VPU)*fU*mAmG	mGQP		Substrate	1299.3	5.1
				Product	868.0	6.0
mU*fU*mA +mG +Phos	mU*fU*mA	mGQP		Substrate	1016.1	5.2
				Product	1455.2	6.3
mC*mU*mAmG +mA +Phos	mC*mU*mAmG	mAQP		Substrate	1310.3	4.9
				Product	866.2	5.9
mUmCmU +mA +Phos	mUmCmU	mAQP		Substrate	896.2	3.7
				Product	1319.2	5.1
mA*fC*mA +fA +Phos	mA*fC*mA	fAQP		Substrate	962.2	4.9 & 5.0 (split peak)
				Product	1373.3	5.9
mA*mA*fGmC +fA +Phos	mA*mA*fGmC	fAQP		Substrate	1321.4	5.4
				Product	865.7	6.4
mA*fC*mAfA +fA +Phos	mA*fC*mAfA	fAQP		Substrate	1293.3	5.3 & 5.5 (split peak)
				Product	851.6	6.3
mUfU*mUfA +mU +Phos	mUfU*mUfA	mUQP		Substrate	1232.2	4.8
				Product	815.6	5.8
(VPU)*fU*mAmG +mG +Phos	(VPU)*fU*mAmG	mGQP		Substrate	687.1	5.2
				Product	906.7	6.1

Table 55.2 HPLC-MS/MS Analysis of Oligonucleotides (7-20mers)

Instrument	Agilent UHPLC 1260 prime series, Agilent 6470B QQQ
Column	Waters Xterra MS C18 50 x 2.1 mm, 5 µm with Phenomenex Security Guard cartridge, C18, 2 mm ID
Mobile phases	A: 15 mM dibutylamine, 25 mM HFIP (hexafluoro-2-propanol) in water; B: methanol; needle wash: 20% methanol in water.
Gradient	Gradient from 90% A to 25% A and back again:

Time (min)	% A
0.0	90
9.0	55
10.0	25
11.5	25
12.0	90
15.0	90

Run time	15.0 min
Flow rate	0.6 mL/min
Peak retention times	Samples typically elute between 5-8 minutes *Note: retention times can shift depending on preparation and age of mobile phase A.*
Column temperature	50 °C
Injection volume	2-5 µL
MS detection conditions	Ion source: Agilent Jet Stream (AJS) ESI; Scan type: MS2 Scan (700-2050 m/z); polarity: negative; scan time: 400 msec; fragmentor voltage: 200 V; cell accelerator voltage: 3 V; time filter window: 0.03 min
MS source conditions	Drying gas temperature: 275°C; drying gas flow: 12.0 L/min; nebulizer pressure: 35 psi; sheath gas temperature: 350°C; sheath gas flow: 12.0 L/min; capillary voltage: -4000 V; nozzle voltage: -2000
Analyte charge state	$[M-3H]^{3-}$

Product Name	Substrate Name	NQP		Analyte	m/z Value	Retention Time (min)
(VPU)*fU*mAmGmGmU(Tgn)mG +mG +Phos	(VPU)*fU*mAmGmGmU(Tgn)mG	mGQP		Substrate	891.4	6.6
				Product	1037.7	7.6
mU*fU*mAmGmGmU(Tgn)mG +mG +Phos	mU*fU*mAmGmGmU(Tgn)mG	mGQP		Substrate	866.0	5.8

Table 55.2 HPLC-MS/MS Analysis of Oligonucleotides (7-20mers)						
				Product	1012.5	7.0

Table 55.3 HPLC-MS/MS Analysis of Oligonucleotides with Chd	
Instrument	Agilent UHPLC 1260 prime series, Agilent 6470B QQQ
Column	Waters Xterra MS C18 50 x 2.1 mm, 5 µm with Phenomenex Security Guard cartridge, C18, 2 mm ID
Mobile Phases	A: 15 mM dibutylamine, 25 mM HFIP (hexafluoro-2-propanol) in water; B: methanol; needle wash: 20% methanol in water.
Gradient C (for oligos with Chd)	Gradient from 80% A to 10% A and back again:

Time (min)	% A
0.0	80
10.0	10
11.5	10
12.0	80
15.0	80

Run time	15.0 min
Flow rate	0.6 mL/min
Peak retention times	Samples typically elute between 7-9 minutes *Note: retention times can shift depending on preparation and age of mobile phase A.*
Column temperature	50 °C
Injection volume	2-5 µL
MS detection conditions	Ion source: Agilent Jet Stream (AJS) ESI; Scan type: MS2 Scan (700-2050 m/z); polarity: negative; scan time: 400 msec; fragmentor voltage: 200 V; cell accelerator voltage: 3 V; time filter window: 0.03 min
MS source conditions	Drying gas temperature: 275°C; drying gas flow: 12.0 L/min; nebulizer pressure: 35 psi; sheath gas temperature: 350°C; sheath gas flow: 12.0 L/min; capillary voltage: -4000 V; nozzle voltage: -2000
Analyte charge state	$[M-3H]^{3-}$

Product Name	Substrate Name	NQP		Analyte	m/z Value	Retention Time (min)
mG*mG*mCmUmA(Chd)mG +mA +Phos	mG*mG*mCmUmA(Chd)mG	mAQP		Substrate	852.1	8.0
				Product	993.3	7.7

Example 56

Improvements over SEQ ID NO: 1928 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0532] SEQ ID NO: 1928 was generated through the removal of Cysteine 390 (C390) through the C390M amino-acid substitution to SEQ ID NO: 1760. SEQ ID NO: 1928 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 56.1**.

[0533] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 56.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11 fUfUfUfC, 5'-6-FAM-T16 *fU*fUfAfU, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T36mCfCmGfG; Concentration labeled oligo (μM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8*fU*fUfAfU, T8mAmUfUmGfG, T8mCfCmGfG; Concentration unlabeled oligo (μM): 2360; Labeled product: 5'-6-FAM-T11 fUfUfUfCmA-3'P, 5'-6-FAM-T16 *fU*fUfAfUmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P; Unlabeled product T8fUfUfUfCmA-3'P, T8*fU*fUfAfUmA-3'P, T8mAmUfUmGfGmA-3'P, T8mCfCmGfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0534] Activity relative to SEQ ID NO: 1928 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1928 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 56.2**.

Table 56.2. Activity relative to SEQ ID NO: 1928		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1928)**	**FIOP activity relative to SEQ ID NO: 1928**
1929/1930	G196Q/S231M/L522V	++
1931/1932	K161G/Q251M/V408A	++
1933/1934	S231M/A416S/M474L	++
1935/1936	A416S	+
1937/1938	R273M/P274R/W325V/A416G/L522V	+

Table 56.2. Activity relative to SEQ ID NO: 1928		
1939/1940	D183T/L522V	+
1941/1942	A416S/M474L	+
1943/1944	R273M	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1928 and are defined as follows: "+" > 1.25, "++" > 1.4.		

Example 57

Improvements over SEQ ID NO: 1944 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0535] SEQ ID NO: 1944 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 57.1**.

[0536] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 57.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T26fUfUfCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mAfUfCfC; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mAmUfUmGfG, T8fUfUfCfG, T8mCfCmGfG, T8mAfUfCfC; Concentration each unlabeled oligo (μM): 2360; Labeled products: 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T26fUfUfCfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P; Unlabeled products: T8mAmUfUmGfGmA-3'P, T8fUfUfCfGmA-3'P, T8mCfCmGfGmA-3'P, T8mAfUfCfCmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0537] Activity relative to SEQ ID NO: 1944 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1944 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 57.2**.

Table 57.2 Activity relative to SEQ ID NO: 1944		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1944)**	**FIOP activity relative to SEQ ID NO: 1944**
1945/1946	S231M/V408A	+
1947/1948	G406P/A463P	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1944 and are defined as follows: "+" > 1.33.		

Example 58

Improvements over SEQ ID NO: 1944 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0538] SEQ ID NO: 1944 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 58.1**.

[0539] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 58.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T36mCfCmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8*fU*fUfAfU, T8mAmUfUmGfG, T8mCfCmGfG; Concentration each unlabeled oligo (µM): 2360; Labeled products: 5'-6-FAM-T11fUfUfUfCmA-3'P, 5'-6-FAM-T16*fU*fUfAfUmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P; Unlabeled products: T8fUfUfUfCmA-3'P, T8*fU*fUfAfUmA-3'P, T8mAmUfUmGfGmA-3'P, T8mCfCmGfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0540] Activity relative to SEQ ID NO: 1944 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1944 (where the percent product may be set as

the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 58.2**.

Table 58.2. Activity relative to SEQ ID NO: 1944		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1944)**	**FIOP activity relative to SEQ ID NO: 1944**
1949/1950	S231N	++
1951/1952	Y424P	++
1953/1954	R429S	++
1955/1956	K430G	++
1957/1958	M499N	+
1959/1960	E367Q	+
1961/1962	M273I	+
1963/1964	M499R	+
1965/1966	M273V	+
1967/1968	E367N	+
1969/1970	P407D	+
1971/1972	L427M	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1944 and are defined as follows: "+" > 1.31, "++" > 1.4.		

Example 59

Improvements over SEQ ID NO: 1946 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0541] SEQ ID NO: 1946 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 59.1**.

[0542] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 59.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 90; Reaction temperature (°C): 50; Reaction Time (min): 30; Reaction volume (µL): 0.1; NTP: NTP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T26fUfUfCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mAfUfCfC; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mAmUfUmGfG, T8fUfUfCfG, T8mCfCmGfG, T8mAfUfCfC; Concentration each unlabeled oligo (µM): 2360; Labeled products: 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T26fUfUfCfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P; Unlabeled products: T8mAmUfUmGfGmA-3'P, T8fUfUfCfGmA-3'P, T8mCfCmGfGmA-3'P, T8mAfUfCfCmA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0543] Activity relative to SEQ ID NO: 1946 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1946 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 59.2**.

Table 59.2. Activity relative to SEQ ID NO: 1946		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1946)**	**FIOP activity relative to SEQ ID NO: 1946**
1973/1974	G196Q/F335V/E367R/K430G	+++
1975/1976	G196Q/M273I/W325V/F335V	+++
1977/1978	G196Q/F335V	+++
1979/1980	G196Q/K430G	+++
1981/1982	G196Q	+++
1983/1984	M273I/W325V/F335V/E367Q	+++
1985/1986	M273I/F335V/K430G	++
1987/1988	F335V	++
1989/1990	P274R/K430G	++
1991/1992	W325V/F335V/C417S/K430G/L479M	++
1993/1994	W325V/F335V/C417S	++
1995/1996	W325V/F335V	++
1997/1998	G196S/F335V/E367R	++
1999/2000	G196S/M273I/W325V/F335V/E367Q	++
2001/2002	F335V/K430G	++
2003/2004	M273I/K430G	++
2005/2006	E367Q	++
2007/2008	E367R/K430G	++
2009/2010	E367R	++
2011/2012	W325V/E367R	++
2013/2014	M273I	++
2015/2016	G196S/E367R/L479M	+
2017/2018	G196S/M273I	+
2019/2020	F335V/L479M	+
2021/2022	F335V/E367Q	+
2023/2024	P274R/W325V/F335V/L479M	+
2025/2026	G196Q/M273I/K430G	+
2027/2028	M273I/E367R	+
2029/2030	G196Q/E367R/L479M	+
2031/2032	G196Q/M273I/W325V	+

Table 59.2. Activity relative to SEQ ID NO: 1946		
2033/2034	M273I/W325V/K430G	+
2035/2036	M273I/K430G/L479M	+
2037/2038	E367Q/L479M	+
2039/2040	C417S/K430G/L479M	+
2041/2042	M273I/P274R/E367R/K430G	+
2043/2044	M273I/P274R	+
2045/2046	F335V/E367R/L479M	+
2047/2048	W325V/E367Q	+
2049/2050	C417S	+
2051/2052	L479M	+
2053/2054	P274R	+
2055/2056	K430G	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1946 and are defined as follows: "+" > 1.23, "++" > 1.45, "+++" > 1.75.		

Example 60

Improvements over SEQ ID NO: 1984 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0544] SEQ ID NO: 1984 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 60.1**.

[0545] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 60.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 90; Reaction temperature (°C): 50; Reaction Time (min): 30; Reaction volume (μL): 0.1; NTP: NTP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T26fUfUfCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mAfUfCfC; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mAmUfUmGfG, T8fUfUfCfG, T8mCfCmGfG, T8mAfUfCfC; Concentration each unlabeled oligo (μM): 2360; Labeled products: 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T26fUfUfCfGmA-3'P, 5'-6-FAM-T36mCfCmGfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P; Unlabeled products: T8mAmUfUmGfGmA-3'P, T8fUfUfCfGmA-3'P, T8mCfCmGfGmA-3'P, T8mAfUfCfCmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0546] Activity relative to SEQ ID NO: 1984 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1984 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 60.2**.

Table 60.2. Activity relative to SEQ ID NO: 1984

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1984)	FIOP activity relative to SEQ ID NO: 1984
2057/2058	M231N/I273K/R380K/C417S/T500N	++
2059/2060	M231N	++
2061/2062	M231N/I273M	+
2063/2064	M231N/I273K	+
2065/2066	M231N/R501K	+
2067/2068	M231N/I273K/P506K	+
2069/2070	M231N/T500N/R501K/P506K	+
2071/2072	M231N/R380K/C417S/R501K	+

Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1984 and are defined as follows: "+" > 1.2, "++" > 1.28.

Example 61

Improvements over SEQ ID NO: 1984 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0547] SEQ ID NO: 1984 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 61.1**.

[0548] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 61.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T16*fA*fG*fAfU, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T26mA*fG*mUmA; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8*fA*fG*fAfU, T8mGfUfAfC, T8mA*fG*mUmA; Concentration each unlabeled oligo (µM): 2000; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAmA-3'P, 5'-6-FAM-T16*fA*fG*fAfUmA-3'P, 5'-6-FAM-T21mGfUfAfCmA-3'P, 5'-6-FAM-T26mA*fG*mUmAmA-3'P; Unlabeled products: T8mU*fA*fG*fAmA-3'P, T8*fA*fG*fAfUmA-3'P, T8mGfUfAfCmA-3'P, T8mA*fG*mUmAmA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0549] Activity relative to SEQ ID NO: 1984 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 1984 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 61.2**.

Table 61.2. activity relative to SEQ ID NO: 1984		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1984)**	**FIOP activity relative to SEQ ID NO: 1984**
2073/2074	I273M	++
2075/2076	W395F	++
2077/2078	V361I	++
2079/2080	P446S	++
2081/2082	V296L	++
2083/2084	L343V	++
2085/2086	V393T	+
2087/2088	T190Q	+
2089/2090	I187Y	+
2091/2092	W395S	+
2093/2094	M390T	+
2095/2096	K384S	+
2097/2098	P446D	+
2099/2100	R260K	+
2101/2102	Q376E	+
2103/2104	M390S	+
2105/2106	V428L	+
2107/2108	L389V	+
2109/2110	N300A	+
2111/2112	C417A	+
2113/2114	R260S	+
2115/2116	Y259F	+
2117/2118	S263R	+
2119/2120	P446G	+
2121/2122	R260T	+
2123/2124	I187L	+
2125/2126	I187V	+
2127/2128	N300K	+
2129/2130	P446T	+

Table 61.2. activity relative to SEQ ID NO: 1984		
2131/2132	L343M	+
2133/2134	T190V	+
2135/2136	A416G	+
2137/2138	P446H	+
2139/2140	I187T	+
2141/2142	Q367E	+
2143/2144	A314E	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 1984 and are defined as follows: "+" > 1.02, "++" > 1.10.		

Example 62

Improvements over SEQ ID NO: 2064 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0550] SEQ ID NO: 2064 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 62.1**.

[0551] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 62.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T26fUfUfUfCfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T46mCfGmUdT, 5'-6-FAM-T56mA*fC*mAfA; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8fUfUfUfCfG, T8mGfUmGfG, T8mCfGmUdT, T8mA*fC*mAfA; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T26fUfUfUfCfGmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P, 5'-6-FAM-T46mCfGmUdTmA-3'P, 5'-6-FAM-T56mA*fC*mAfAmA-3'P; Unlabeled products: T8fUfUfUfCfGmA-3'P, T8mGfUmGfGmA-3'P, T8mCfGmUdTmA-3'P, T8mA*fC*mA5fAmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0552] Activity relative to SEQ ID NO: 2064 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2064 (where the percent product may be set as

the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 62.2**.

Table 62.2. Activity relative to SEQ ID NO: 2064		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2064)**	**FIOP activity relative to SEQ ID NO: 2064**
2145/2146	R318K	++
2147/2148	V174W	++
2149/2150	A201Q	++
2151/2152	V525T	+
2153/2154	K161L	+
2155/2156	V244R	+
2157/2158	I503V	+
2159/2160	A252L	+
2161/2162	L254Q	+
2163/2164	L254R	+
2165/2166	T437H	+
2167/2168	E246L	+
2169/2170	T162P	+
2171/2172	D223H	+
2173/2174	H433A	+
2175/2176	R219D	+
2177/2178	L254N	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2064 and are defined as follows: "+" > 1.1, "++" > 1.18.		

Example 63

Improvements over SEQ ID NO: 2104 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0553] SEQ ID NO: 2104 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 63.1**.

[0554] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 63.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 67.5; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mAmUmCmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mAmUmCmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0555] Activity relative to SEQ ID NO: 2104 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2104 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 63.2**.

Table 63.2. Activity relative to SEQ ID NO: 2104		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2104)**	**FIOP activity relative to SEQ ID NO: 2104**
2179/2180	Y259F/S263R/I273K/L389V/S390T	+++
2181/2182	I273K/L389V/P446S	+++
2183/2184	V174L/Y259F/I273K/L343V/V361I/L389V	+++
2185/2186	G196Q/D199V/M231N/S263T/I273K/L389V	+++
2187/2188	V174L/M231N/S390T	+++
2189/2190	V174L/M231N/S263T/I273K/L343V	+++
2191/2192	V174L/M231N/R260K/I273K/L389V/S390T	++
2193/2194	M231N/I273K	++
2195/2196	I187V/M231N/N300K/L353V	++
2197/2198	D199V/M231N/R260K/S263T/V361I/L389V/P446S	++
2199/2200	M231N/Y259F/S263T/I273K	++
2201/2202	V174L/Y259F/R260K/S263R/I273K/V361I/L389V/S390T/P446S	++
2203/2204	V174L/I273K/L343V/L389V/S390T/P446S	++
2205/2206	I273K/S390T	++
2207/2208	I187Y/T190Q/M231N/A252M/I273K/V296L/N300K/L353V/C417S	+
2209/2210	I187Y/M231N/V296L/N300K/L353R	+
2211/2212	V174L/M231N/L389V/S390T/P446S	+
2213/2214	V174L/M231N	+
2215/2216	I187V/M231N/A252E/I273K/V296L/N300K/L353R	+
2217/2218	T190Q/M231N/I273K	+
2219/2220	I273K	+
2221/2222	V174L/M231N/S263T/L389V	+
2223/2224	V174L/M231N/S263R/L389V	+
2225/2226	I187V/T190Q/A252M/I273K/N300K/C417S	+
2227/2228	I187Y/M231N/I273K/V296L/N300K/L353R	+
2229/2230	I187V/T190Q/I273K/L353V	+
2231/2232	S263T/I273K/L343V/P446S	+
2233/2234	V174L/S263R/L389V/S390T	+
2235/2236	R260K/I273K/P446S	+
2237/2238	I273K/L353V	+
2239/2240	I187V/I273K/L353V	+
2241/2242	V174L/D199V/L389V/S390T	+
2243/2244	M231N	+

Table 63.2. Activity relative to SEQ ID NO: 2104
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2104 and are defined as follows: "+" > 8.0, "++" > 14.0, "+++" > 20.0.

Example 64

Improvements over SEQ ID NO: 2210 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0556] SEQ ID NO: 2210 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 64.1**.

[0557] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 64.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T21*mAfAfAfA, 5'-6-FAM-T31mC*fGmAfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T51mU*fA*fAfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8*mAfAfAfA, T8mC*fGmAfG, T8mGfUmGfG, T8mU*fA*fAfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T21*mAfAfAfAfA-3'P, 5'-6-FAM-T31mC*fGmAfGfA-3'P, 5'-6-FAM-T41mGfUmGfGfA-3'P, 5'-6-FAM-T51mU*fA*fAfGfA-3'P; Unlabeled products: T8*mAfAfAfA, T8mC*fGmAfG, T8mGfUmGfG, T8mU*fA*fAfG; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0558] Activity relative to SEQ ID NO: 2210 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2210 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 64.2**.

Table 64.2. Activity relative to SEQ ID NO: 2210		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2210)**	**FIOP activity relative to SEQ ID NO: 2210**
2245/2246	A201Q/Q376E	++
2247/2248	K161L/T162P/R318K/P446S	++
2249/2250	K161L/T162P/L254R/C417A	++
2251/2252	V174W/V244G/T437H/V525T	+
2253/2254	H433P	+
2255/2256	V174W/I273K/I503L	+
2257/2258	V174W/V244R/I273K/S390T/T437H/I503L	+
2259/2260	V525T	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2210 and are defined as follows: "+" > 1.0, "++" > 1.18.		

Example 65

Improvements over SEQ ID NO: 2210 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0559] SEQ ID NO: 2210 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 65.1**.

[0560] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 65.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T16*fC*mAfAfA, 5'-6-FAM-T26fUfUfCfG, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T51mU*fA*fAfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8*fC*mAfAfA, T8fUfUfCfG, T8mAfUfCfC, T8mU*fA*fAfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T16*fC*mAfAfAmA-3'P, 5'-6-FAM-T26fUfUfCfGmA-3'P, 5'-6-FAM-T41mAfUfCfCmA-3'P, 5'-6-FAM-T51mU*fA*fAfGmA-3'P; Unlabeled products: T8*fC*mAfAfAmA-3'P, T8fUfUfCfGmA-3'P, T8mAfUfCfCmA-3'P, T8mU*fA*fAfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0561] Activity relative to SEQ ID NO: 2210 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2210 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 65.2**.

Table 65.2. Activity relative to SEQ ID NO: 2210		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2210)**	**FIOP activity relative to SEQ ID NO: 2210**
2261/2262	K397S	+
2263/2264	M466I	+
2265/2266	K303E	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2210 and are defined as follows: "+" > 1.02.		

Example 66

Improvements over SEQ ID NO: 2210 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0562] SEQ ID NO: 2210 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 66.1**.

[0563] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 66.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fUTP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T46mCfGmUdT; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8mCfGmUdT; Concentration each unlabeled oligo (µM): 3975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAfU-3'P, 5'-6-FAM-T46mCfGmUdTfU-3'P, 0, 0; Unlabeled products: T8mU*fA*fG*fAfU-3'P, T8mCfGmUdTfU-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0564] Activity relative to SEQ ID NO: 2210 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2210 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 66.2**.

Table 66.2. Activity relative to SEQ ID NO: 2210

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2210)	FIOP activity relative to SEQ ID NO: 2210
2261/2262	K397S	++
2265/2266	K303E	+
2267/2268	V290L	+
2269/2270	S297L	+
2271/2272	V525G	+
2273/2274	K278S	+
2275/2276	K397G	+
2277/2278	S297Y	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2210 and are defined as follows: "+" > 1.04, "++" > 1.1.		

Example 67

Improvements over SEQ ID NO: 2256 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0565] SEQ ID NO: 2256 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 67.1**.

[0566] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 67.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 30; Reaction volume (µL): 1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T16*fA*fG*fAfU, 5'-6-FAM-T21*mAfAfAfA, 5'-6-FAM-T20mAmGfAmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fA, T8*fA*fG*fAfU, T8*mAfAfAfA, T8mAmGfAmGfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fAmA-3'P, 5'-6-FAM-T16*fA*fG*fAfUmA-3'P, 5'-6-FAM-T21*mAfAfAfAmA-3'P, 5'-6-FAM-T20mAmGfAmGfGmA-3'P; Unlabeled products: T8mU*fA*fAmA-3'P, T8*fA*fG*fAfUmA-3'P, T8*mAfAfAfAmA-3'P, T8mAmGfAmGfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0567] Activity relative to SEQ ID NO: 2256 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2256 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 67.2**.

Table 67.2 Activity relative to SEQ ID NO: 2256		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2256)**	**FIOP activity relative to SEQ ID NO: 2256**
2279/2280	T162P/L254R/Q376E/C417A/H433P	+++
2281/2282	T162P/A201Q/R318K/Q376E/C417A/H433P/V525T	+++
2283/2284	F22P/R318K/Q376E/P446S	+++
2285/2286	T162P/L254R/R318K/Q376E/C417A/H433P	+++
2287/2288	T162P/Q376E/V525T	++
2289/2290	F22P/Q376E/C417A/T437H/P446S/V525T	++
2291/2292	F22P/T162P/A201Q/V244G/L254R/R318K/Q376E/C417A/H433P /T437H/V525T	++
2293/2294	T162P/V244G/L254R/H433P	++
2295/2296	T162P/V244G/R318K/Q376E/C417A/H433P/T437H	++
2297/2298	V244G/R318K/Q376E/C417A/H433P/T437H/V525T	++
2299/2300	R318K/Q376E	++
2301/2302	A201Q/V244G/R318K/Q376E	++
2303/2304	V244G/R318K/Q376E/C417A	++
2305/2306	K161L/T162P/A201Q/V244G/R318K/Q376E/C417A/T437H	++
2307/2308	H433P	+
2309/2310	F22P/A201Q/Q376E	+
2311/2312	K161L/T162P/A201Q/V244G/Q376E	+
2313/2314	F22P/V244G	+
2315/2316	A201Q/R318K/Q376E/P446S	+
2317/2318	T437H	+
2319/2320	F22P/R318K/Q376E/H433P/V525T	+
2321/2322	R318K/Q376E/H433P	+
2323/2324	F22P	+
2325/2326	F22P/V244G/L254R	+
2327/2328	F22P/V244G/H433P/V525T	+
2329/2330	T162P	+
2331/2332	T162P/A201Q/R318K/Q376E/H433P/T437H/P446S	+
2333/2334	A201Q/V244G/Q376E/H433P/V525T	+
2335/2336	T162P/L254R	+

Table 67.2 Activity relative to SEQ ID NO: 2256		
2337/2338	T162P/H433P	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2256 and are defined as follows: "+" > 1.0, "++" > 1.2, "+++" > 1.3.		

Example 68

Improvements over SEQ ID NO: 2280 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0568] SEQ ID NO: 2280 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 68.1**.

[0569] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 68.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mUTP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fA , 5'-6-FAM-T21mGfUfAfC , 5'-6-FAM-T41mGfUmGfG , 5'-6-FAM-T46mCfGmUdT ; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fA , T8mGfUfAfC , T8mGfUmGfG , T8mCfGmUdT ; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fAmU-3'P, 5'-6-FAM-T21mGfUfAfCmU-3'P, 5'-6-FAM-T41mGfUmGfGmU-3'P, 5'-6-FAM-T46mCfGmUdTmU-3'P; Unlabeled products: T8mU*fA*fAmU-3'P, T8mGfUfAfCmU-3'P, T8mGfUmGfGmU-3'P, T8mCfGmUdTmU-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0570] Activity relative to SEQ ID NO: 2280 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2280 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 68.2**.

Table 68.2. Activity relative to SEQ ID NO: 2280		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2280)**	**FIOP activity relative to SEQ ID NO: 2280**
2339/2340	I41T/K42T/F43-/K397S	++
2341/2342	F22P/D34Y/K278S/R318K/T437H/M466I	+
2343/2344	F43T/V244G/K397S	+
2345/2346	F22P/M466I	+
2347/2348	K397S	+
2349/2350	F22P/Q27P/T437H/M466I	+
2351/2352	E45T/K397S	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2280 and are defined as follows: "+" > 1.1, "++" > 1.2.		

Example 69

Improvements over SEQ ID NO: 2280 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0571] SEQ ID NO: 2280 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 69.1**.

[0572] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 69.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 67.5; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0573] Activity relative to SEQ ID NO: 2280 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2280 (where the percent product may be set as

the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 69.2**.

Table 69.2. Activity relative to SEQ ID NO: 2280		
SEQ ID NO: (nt/aa)	**Amino Acid Differences](Relative to SEQ ID NO: 2280)**	**FIOP activity relative to SEQ ID NO: 2280**
2353/2354	N176H	+++
2355/2356	Q179K	+++
2357/2358	S175R	+++
2359/2360	S175L	+++
2361/2362	S263R	+++
2363/2364	V328A	++
2365/2366	Q477L	++
2367/2368	T173P	++
2369/2370	Q179R	++
2371/2372	R480K	++
2373/2374	P274L	++
2375/2376	M197L	++
2377/2378	L462Y	++
2379/2380	S277D	++
2381/2382	V319A	++
2383/2384	E376T	++
2385/2386	S263T	++
2387/2388	G196D	+
2389/2390	H308Y	+
2391/2392	Q477W	+
2393/2394	Q405L	+
2395/2396	L388V	+
2397/2398	Q179C	+
2399/2400	A408G	+
2401/2402	E310S	+
2403/2404	V341T	+
2405/2406	T182I	+
2407/2408	Q208T	+
2409/2410	G411T	+
2411/2412	K273M	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2280 and are defined as follows: "+" > 1.1, "++" > 1.3, "+++" > 2.0.		

Example 70

Improvements over SEQ ID NO: 2280 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0574] SEQ ID NO: 2280 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 70.1**.

[0575] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 70.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mGfU*mC*fA, 5'-6-FAM-T16*fA*fG*fAfU, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T30mAfGfUmGfG; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mGfU*mC*fA, T8*fA*fG*fAfU, T8mAmG5fAmGfG, T8mAfGfUmGfG; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T11mGfU*mC*fAmA-3'P, 5'-6-FAM-T16*fA*fG*fAfUmA-3'P, 5'-6-FAM-T20mAmGfAmGfGmA-3'P, 5'-6-FAM-T30mAfGfUmGfGmA-3'P; Unlabeled products: T8mGfU*mC*fA, T8*fA*fG*fAfU, T8mAmGfAmGfG, T8mAfGfUmGfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0576] Activity relative to SEQ ID NO: 2280 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2280 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 70.2**.

Table 70.2. Activity relative to SEQ ID NO: 2280		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2280)**	**FIOP activity relative to SEQ ID NO: 2280**
2373/2374	P274L	+
2379/2380	S277D	++
2391/2392	Q477W	+
2411/2412	K273M	+
2413/2414	S299G	+
2415/2416	K273Q	+
2417/2418	K273G	+
2419/2420	I324T	+
2421/2422	K278D	+
2423/2424	R291L	+
2425/2426	K273N	+
2427/2428	K300S	+
2429/2430	K195T	+
2431/2432	K273E	+
2433/2434	S299H	+
2435/2436	M203Q	+

Table 70.2. Activity relative to SEQ ID NO: 2280
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2280 and are defined as follows: "+" > 1.1, "++" > 1.3, "+++" > 2.0.

Example 71

Improvements over SEQ ID NO: 2342 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0577] SEQ ID NO: 2342 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 71.1**.

[0578] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 71.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: mAQP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mGfU*mC*fA, 5'-6-FAM-T16mGmAmCfC, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T26fUfUfCfG; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mGfU*mC*fA, T8mGmAmCfC, T8mAmUfUmGfG, T8fUfUfCfG; Concentration each unlabeled oligo (μM): 1580; Labeled products: 5'-6-FAM-T11mGfU*mC*fAmA-3'P, 5'-6-FAM-T16mGmAmCfCmA-3'P, 5'-6-FAM-T20mAmUfUmGfGmA-3'P, 5'-6-FAM-T26fUfUfCfGmA-3'P; Unlabeled products: T8mGfU*mC*fAmA-3'P, T8mGmAmCfCmA-3'P, T8mAmUfUmGfGmA-3'P, T8fUfUfCfGmA-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0579] Activity relative to SEQ ID NO: 2342 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2342 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 71.2.**

Table 71.2. Activity relative to SEQ ID NO: 2342		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2342)**	**FIOP activity relative to SEQ ID NO: 2342**
2437/2438	K300S/S396T/Q477L/R480K	+
2439/2440	Y34D/S396T	+
2441/2442	Y34D/K273M/K300S/Q477L/R480K	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2342 and are defined as follows: "+" > 3.9.		

Example 72

Improvements over SEQ ID NO: 2438 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0580] SEQ ID NO: 2438 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 72.1**.

[0581] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 72.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 68; Preincubation Time (min): 90; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: fCTP-3'P; NTP conc (μM): 4000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (μM): 1975; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0582] Activity relative to SEQ ID NO: 2438 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2438 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 72.2**.

Table 72.2. Activity relative to SEQ ID NO: 2438		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2438)**	**FIOP activity relative to SEQ ID NO: 2438**
2443/2444	T173P/N176H/Q179R	+++
2445/2446	T173P/Q179R	+++
2447/2448	N176H/Q179R/M197L	+++
2449/2450	N176H/Q179R	+++
2451/2452	T173P/N176H/Q179K/S277D	+++
2453/2454	Q27P/T173P/Q179K/K273M	+++
2455/2456	Q179R	+++
2457/2458	Q27P/Y34D/T173P/N176H/Q179K/K195T/S277D	++
2459/2460	Q179K	++
2461/2462	Q27P/T173P/N176H	++
2463/2464	T173P/Q179R/K273M/S277D	++
2465/2466	Q27P/T173P/N176H/Q179K/K273M/S277D	++
2467/2468	Q27P/T173P/Q179K/S277D	++
2469/2470	T173P/N176H/Q179R/M197L	++
2471/2472	Q27P/T173P/N176H/Q179R/S277D	++
2473/2474	N176H/Q179K/K273M	++
2475/2476	T173P/Q179R/K273M	++
2477/2478	N176H/Q179K/K273M/S277D	++
2479/2480	Q27P/N176H/Q179R/K273M	++
2481/2482	Y34D/T173P/N176H/M197L/S277D	++
2483/2484	Q27P/Q179K	+
2485/2486	Q27P/T173P/Q179K/K273M/S277D	+
2487/2488	T173P/Q179K/K273M/S277D	+
2489/2490	Q179K/M197L	+
2491/2492	Q27P/Q179R/S277D	+
2493/2494	E105G/T173P/N176H/M197L/S277D	+
2495/2496	Q179R/M197L	+
2497/2498	Q179K/K273M	+
2499/2500	T173P/S277D	+
2501/2502	Q27P/T173P	+
2503/2504	N176H/Q179R/K273M/S277D	+
2505/2506	Q179K/K273M/S277D	+
2507/2508	Q27P/Q179K/K273M/S277D	+
2509/2510	Y34D	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2438 and are defined as follows: "+" > 1.7, "++" > 7.0, "+++" > 8.9.		

Example 73

Improvements over SEQ ID NO: 2438 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0583] SEQ ID NO: 2438 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 73.1**.

[0584] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and

1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 73.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (μL): 0.1; NTP: mUTP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T16mGmAmCfC, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T31mUmUmGfU; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8mGmAmCfC, T7mAmGfAmGfG, T8mUmUmGfU; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAmU-3'P, 5'-6-FAM-T16mGmAmCfCmU-3'P, 5'-6-FAM-T20mAmGfAmGfGmU-3'P, 5'-6-FAM-T31mUmUmGfUmU-3'P; Unlabeled products: T8mU*fA*fG*fAmU-3'P, T8mGmAmCfCmU-3'P, T7mAmGfAmGfGmU-3'P, T8mUmUmGfUmU-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0585] Activity relative to SEQ ID NO: 2438 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2438 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 73.2**.

Table 73.2. Activity relative to SEQ ID NO: 2438

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2438)	FIOP activity relative to SEQ ID NO: 2438
2511/2512	E376H	+++
2513/2514	K487R	+++
2515/2516	K430S	+++
2517/2518	G406D	++
2519/2520	G406P	++
2521/2522	K508C	++
2523/2524	D199E	++
2525/2526	V339T	++
2527/2528	P352A	++
2529/2530	P352T	++
2531/2532	R177C	++
2533/2534	E342T	+
2535/2536	K430G	+
2537/2538	W229L	+
2539/2540	V525T	+
2541/2542	S292A	+
2543/2544	L202V	+
2545/2546	P407N	+
2547/2548	P363G	+

Table 73.2. Activity relative to SEQ ID NO: 2438
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2438 and are defined as follows: "+" > 1.0, "++" > 1.12, "+++" > 1.18.

Example 74

Improvements over SEQ ID NO: 2452 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0586] SEQ ID NO: 2452 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 74.1**.

[0587] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 74.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 71; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 60; Reaction volume (μL): 0.1; NTP: fCTP-3'P; NTP conc (μM): 4000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (μM): 1975; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (μM): 1.

[0588] Activity relative to SEQ ID NO: 2452 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2452 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 74.2**.

Table 74.2. Activity relative to SEQ ID NO: 2452		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2452)**	**FIOP activity relative to SEQ ID NO: 2452**
2549/2550	D183R/R205L	++
2551/2552	D183R	++
2553/2554	H176N/D183R/R205L	++

Table 74.2. Activity relative to SEQ ID NO: 2452		
2555/2556	D183G/R205L	+
2557/2558	D183G	+
2559/2560	D183R/R205L/A408V	+
2561/2562	R205L	+
2563/2564	H176N/D183R	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2452 and are defined as follows: "+" > 1.5, "++" > 2.6.		

Example 75

Improvements over SEQ ID NO: 2562 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0589] SEQ ID NO: 2562 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 75.1**.

[0590] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 75.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: mUTP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T16mGmAmCfC, 5'-6-FAM-T26fUfUfCfG, 5'-6-FAM-T36mAmAfGmA, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fUfUfUfC, T8fUfUfCfG, T8mAmAfGmA, T8mGfUmGfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T16mGmAmCfCmU-3'P, 5'-6-FAM-T26fUfUfCfGmU-3'P, 5'-6-FAM-T36mAmAfGmAmU-3'P, 5'-6-FAM-T41mGfUmGfGmU-3'P; Unlabeled products: T8fUfUfUfCmU-3'P, T8fUfUfCfGmU-3'P, T8mAmAfGmAmU-3'P, T8mGfUmGfGmU-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0591] Activity relative to SEQ ID NO: 2562 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2562 (where the percent product may be set as

the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 75.2**.

Table 75.2. Activity relative to SEQ ID NO: 2562		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2562)**	**FIOP activity relative to SEQ ID NO: 2562**
2565/2566	S292A/V339T/E342T/E376H/G406P/K487R	++
2567/2568	R177K/S292A/V339T/G406D	++
2569/2570	S292A/E342T/G406D	++
2571/2572	V339T/E342T/P407N	+
2573/2574	V339T/E342T	+
2575/2576	R177K/V339T/E342T/G406P/K487R	+
2577/2578	R177K/V339T/E342T/E376H	+
2579/2580	V339T/E342T/P407N/K487R	+
2581/2582	R177K/S292A/E342T/G406P	+
2583/2584	R177K/K487R	+
2585/2586	R177K/S292A/V339T/E342T/G406D	+
2587/2588	S292A/E342T	+
2589/2590	R177K/V339T/E342T	+
2591/2592	R177K/V339T/E342T/G406D	+
2593/2594	G406P	+
2595/2596	R177K/S292A/E342T/P363G/E376H/G406D	+
2597/2598	R177K/E342T/G406P/K430G	+
2599/2600	R177K/P407N	+
2601/2602	S292A/G406P	+
2603/2604	R177K/S292A/V339T/G406P	+
2605/2606	R177K/E342T/E376H/P407N	+
2607/2608	R177K	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2562 and are defined as follows: "+" > 1.1, "++" > 1.3.		

Example 76

Improvements over SEQ ID NO: 2562 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0592] SEQ ID NO: 2562 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 76.1**.

[0593] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then

held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 76.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 90; Reaction volume (µL): 0.1; NTP: mUTP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T16mGmAmCfC, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8mGmAmCfC, T8mUfCmAfU, T8mGfUmGfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAmU-3'P, 5'-6-FAM-T16mGmAmCfCmU-3'P, 5'-6-FAM-T31mUfCmAfUmU-3'P, 5'-6-FAM-T41mGfUmGfGmU-3'P; Unlabeled products: T8mU*fA*fG*fAmU-3'P, T8mGmAmCfCmU-3'P, T8mUfCmAfUmU-3'P, T8mGfUmGfGmU-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0594] Activity relative to SEQ ID NO: 2562 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2562 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 76.2.**

Table 76.2. Activity relative to SEQ ID NO: 2562		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2562)**	**FIOP activity relative to SEQ ID NO: 2562**
2609/2610	S402P/P407A	+++
2611/2612	D198T	+++
2613/2614	L379M	++
2615/2616	T190G	++
2617/2618	D412P	++
2619/2620	L359F	++
2621/2622	W174I	++
2623/2624	D198P	++
2625/2626	E414Q	++
2627/2628	Y187T	++
2629/2630	D277S	+
2631/2632	T307K	+
2633/2634	P274V	+
2635/2636	L343R	+
2637/2638	H494Y	+
2639/2640	S267G	+
2641/2642	D198S	+
2643/2644	P407S	+
2645/2646	E523H	+
2647/2648	H494T	+
2649/2650	V316L	+
2651/2652	D409S	+
2653/2654	E523A	+
2655/2656	D481H	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2562 and are defined as follows: "+" > 1.2, "++" > 1.4, "+++" > 1.6.		

Example 77

Improvements over SEQ ID NO: 2576 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0595] SEQ ID NO: 2576 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 77.1**.

[0596] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 77.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 75; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mAmUmCmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mAmUmCmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0597] Activity relative to SEQ ID NO: 2576 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2576 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 77.2**.

Table 77.2. Activity relative to SEQ ID NO: 2576		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2576)**	**FIOP activity relative to SEQ ID NO: 2576**
2657/2658	S263R	+++
2659/2660	K235R	+++
2661/2662	P406E	+++
2663/2664	M238L	++
2665/2666	L477I	++
2667/2668	T224V	++
2669/2670	L221I	++

Table 77.2. Activity relative to SEQ ID NO: 2576		
2671/2672	L202M	++
2673/2674	M238I	++
2675/2676	R487K	++
2677/2678	E239S	++
2679/2680	P406T	++
2681/2682	E249Q	++
2683/2684	A252R	++
2685/2686	P406M	++
2687/2688	Y259F	++
2689/2690	R484Y/R487K	++
2691/2692	Q251A	+
2693/2694	I242L	+
2695/2696	G236R	+
2697/2698	E239G	+
2699/2700	R484L/R487K	+
2701/2702	E239R	+
2703/2704	Q208M	+
2705/2706	Q405V/P406G	+
2707/2708	Q405G/P406G	+
2709/2710	E249L	+
2711/2712	E239L	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2576 and are defined as follows: "+" > 1.3, "++" > 1.6, "+++" > 2.8.		

Example 78

Improvements over SEQ ID NO: 2576 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0598] SEQ ID NO: 2576 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 78.1**.

[0599] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 78.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T26fAfAmGfC, 5'-6-FAM-T31mUmUmGfU, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8fAfAmGfC, T8mUmUmGfU, T8mGfUmGfG; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAmA-3'P, 5'-6-FAM-T26fA5'-6-FAM-GfCmA-3'P, 5'-6-FAM-T31mUmUmGfUmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P; Unlabeled products: T8mU*fA*fG*fAmA-3'P, T8fAfAmGfCmA-3'P, T8mUmUmGfUmA-3'P, T8mGfUmGfGmA-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0600] Activity relative to SEQ ID NO: 2576 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2576 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 78.2**.

Table 78.2 Activity relative to SEQ ID NO: 2576		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2576)**	**FIOP activity relative to SEQ ID NO: 2576**
2713/2714	W174I/F204M/G236R/R254I/R353V	+++
2715/2716	W174I/T190G/L202H/F204M/R254I	+++
2717/2718	W174I/L202H/F204M/R254I/R353V	+++
2719/2720	Y187T	++
2721/2722	Y187T/T224V/A321R/P436S	++
2723/2724	W174I/T190G/L202H/R254I	++
2725/2726	W174I/T190G/F204M	++
2727/2728	F204V/R254I/R353V	++
2729/2730	Y187T/E249M/A321R/L379M/P436S	++
2731/2732	L359F/P407S/D412P	++
2733/2734	L379M	++
2735/2736	L359F/P407A	+
2737/2738	P436S	+
2739/2740	W174I/T190G	+
2741/2742	W174I/T190G/G236R	+
2743/2744	Y187T/E249M	+
2745/2746	Y187T/T224V/L379M/P436S	+
2747/2748	Y187T/T224V	+
2749/2750	E249M/L379M	+
2751/2752	W174I/L202H/F204M/R254I	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2576 and are defined as follows: "+" > 1.2, "++" > 1.26, "+++" > 1.35.		

Example 79

Improvements over SEQ ID NO: 2576 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0601] SEQ ID NO: 2576 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 79.1**.

[0602] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 79.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 72; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0603] Activity relative to SEQ ID NO: 2576 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2576 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 79.2**.

Table 79.2. Activity relative to SEQ ID NO: 2576		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2576)**	**FIOP activity relative to SEQ ID NO: 2576**
2753/2754	K392Q	++
2755/2756	R313Q	++
2757/2758	K318P	++
2759/2760	K392S	+
2761/2762	S293R	+
2763/2764	S300R	+
2765/2766	I340T	+
2767/2768	E310D	+
2769/2770	R219Q	+
2771/2772	P364S	+
2773/2774	I340L	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2576 and are defined as follows: "+" > 1.05, "++" > 1.16.		

Example 80

Improvements over SEQ ID NO: 2576 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0604] SEQ ID NO: 2576 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 80.1**.

[0605] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 80.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (μL): 0.1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T16 *fU*fUfAfU, 5'-6-FAM-T21mA*fG*mU, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8*fU*fUfAfU, T8mA*fG*mU, T8mUfCmAfU, T8mGfUmGfG; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T16 *fU*fUfAfUmA-3'P, 5'-6-FAM-T21mA*fG*mUmA-3'P, 5'-6-FAM-T31mUfCmAfUmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P; Unlabeled products: T8*fU*fUfAfUmA-3'P, T8mA*fG*mUmA-3'P, T8mUfCmAfUmA-3'P, T8mGfUmGfGmA-3'P; IPP: SEQ ID NO: 1926; IPP (μM): 1.

[0606] Activity relative to SEQ ID NO: 2576 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2576 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 80.2**.

Table 80.2. Activity relative to SEQ ID NO: 2576		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2576)**	**FIOP activity relative to SEQ ID NO: 2576**
2757/2758	K318P	+
2761/2762	S293R	++
2763/2764	S300R	++

Table 80.2. Activity relative to SEQ ID NO: 2576		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2576)**	**FIOP activity relative to SEQ ID NO: 2576**
2767/2768	E310D	+
2775/2776	S175P	+++
2777/2778	P173H	+++
2779/2780	E376A	+++
2781/2782	E376G	+++
2783/2784	E376S	+++
2785/2786	E376R	++
2787/2788	T339A	++
2789/2790	W301F	++
2791/2792	S175H	++
2793/2794	A366P	++
2795/2796	S278A	++
2797/2798	S278L	++
2799/2800	K318S	++
2801/2802	K161L	++
2803/2804	V319P	++
2805/2806	M160A	+
2807/2808	M160T	+
2809/2810	S368R	+
2811/2812	K318R	+
2813/2814	T339S	+
2815/2816	T342S	+
2817/2818	A366H	+
2819/2820	V244I	+
2821/2822	V244S	+
2823/2824	V378M	+
2825/2826	A366F	+
2827/2828	E376V	+
2829/2830	Q370L	+
2831/2832	Q367V	+
2833/2834	I324A	+
2835/2836	T334S	+
2837/2838	A222N	+
2839/2840	M160D	+
2841/2842	K161A	+
2843/2844	V341L	+
2845/2846	Q370R	+
2847/2848	M160W	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2576 and are defined as follows: "+" > 1.12, "++" > 1.9, "+++" > 3.2.		

Example 81

Improvements over SEQ ID NO: 2714 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0607] SEQ ID NO: 2714 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of

previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 81.1**.

[0608] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 81.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 71; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T46mUmCmUmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mUmCmUmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T46mUmCmUmUfC-3'P; Unlabeled product T8mUmCmUmUfC-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0609] Activity relative to SEQ ID NO: 2714 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2714 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 81.2**.

Table 81.2. Activity relative to SEQ ID NO: 2714		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2714)**	**FIOP activity relative to SEQ ID NO: 2714**
2849/2850	Y187T/S263R	+++
2851/2852	Y187T/I254R/S263R/L359F/L379M/P407S	+++
2853/2854	Y187T/T224V/S263R	+++
2855/2856	E249M/S263R	++
2857/2858	Y187T/T224V	++
2859/2860	S263R	++
2861/2862	Y187T/T224V/P407A	++
2863/2864	Y187T/L379M	++
2865/2866	S263R/P407S/D412P	++
2867/2868	E249M/S263R/P407S	+
2869/2870	Y187T	+
2871/2872	Y187T/L359F	+
2873/2874	Y187T/T224V/D412P	+
2875/2876	Y187T/T224V/P407A/D412P	+
2877/2878	T224V/L359F/P407A	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2714 and are defined as follows: "+" > 1.3, "++" > 1.6, "+++" > 2.6.		

Example 82

Improvements over SEQ ID NO: 2876 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0610] SEQ ID NO: 2876 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 82.1**.

[0611] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 82.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (μL): 0.1; NTP: mATP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T26fAfAmGfC, 5'-6-FAM-T31mUmUmGfU, 5'-6-FAM-T41mGfUmGfG; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8fAfAmGfC, T8mUmUmGfU, T8mGfUmGfG; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAmA-3'P, 5'-6-FAM-T26fA5'-6-FAM-GfCmA-3'P, 5'-6-FAM-T31mUmUmGfUmA-3'P, 5'-6-FAM-T41mGfUmGfGmA-3'P; Unlabeled products: T8mU*fA*fG*fA, T8fAfAmGfC, T8mUmUmGfU, T8mGfUmGfG; IPP: SEQ ID NO: 1926; IPP (μM): 1.

[0612] Activity relative to SEQ ID NO: 2876 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2876 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 82.2**.

Table 82.2. Activity relative to SEQ ID NO: 2876		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2876)**	**FIOP activity relative to SEQ ID NO: 2876**
2879/2880	E310D/K318P/I340T	++
2881/2882	S263R/K318P	++
2883/2884	M160T/S263R/K318P/T339S	++

Table 82.2. Activity relative to SEQ ID NO: 2876		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2876)**	**FIOP activity relative to SEQ ID NO: 2876**
2885/2886	S293R/T334S/P364S/A407P	++
2887/2888	S263R/E310D/K318S/A366H	+
2889/2890	T334S/P364S	+
2891/2892	K318P	+
2893/2894	V244S/S293R/T334S	+
2895/2896	R219S/E310D/I340T	+
2897/2898	S175H/V244I/S293R/P364S/A407P	+
2899/2900	R219S/E310D	+
2901/2902	S293R/R313Q/T334S/P364S	+
2903/2904	V244I/R313Q	+
2905/2906	V244I/R313Q/T334S/P364S/A407S	+
2907/2908	V244I/S293R/R313Q	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2876 and are defined as follows: "+" > 1.32, "++" > 1.4.		

Example 83

Improvements over SEQ ID NO: 2884 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0613] SEQ ID NO: 2884 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 83.1**.

[0614] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 83.1
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T16*fA*fG*fAfU, 5'-6-FAM-T26 mAfGfGfC, 5'-6-FAM-T31mUfCmAfU; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mU*fA*fA, T8*fA*fG*fAfU, T8mAfGfGfC, T8mUfCmAfU; Concentration each unlabeled oligo (µM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fAmA-3'P, 5'-6-FAM-T16*fA*fG*fAfUmA-3'P, 5'-6-FAM-T26 mAfGfGfCmA-3'P, 5'-6-FAM-T31mUfCmAfUmA-3'P; Unlabeled products: T8mU*fA*fAmA-3'P, T8*fA*fG*fAfUmA-3'P, T8mAfGfGfCmA-3'P, T8mUfCmAfUmA-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0615] Activity relative to SEQ ID NO: 2884 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2884 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 83.2**.

Table 83.2. Activity relative to SEQ ID NO: 2884		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2884)**	**FIOP activity relative to SEQ ID NO: 2884**
2909/2910	E310D/T334S	++
2911/2912	I340T	++
2913/2914	I254R/S293R/E310D	+
2915/2916	I254R/S293R/E310D/T334S/I340T/A407P	+
2917/2918	T334S	+
2919/2920	A407P	+
2921/2922	T334S/A407P	+
2923/2924	T334S/I340T	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2884 and are defined as follows: "+" > 1.2, "++" > 1.26.		

Example 84

Improvements over SEQ ID NO: 2884 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0616] SEQ ID NO: 2884 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 84.1**.

[0617] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for

TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 84.1
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 69.5; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mAmUmCmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mAmUmCmUfC-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0618] Activity relative to SEQ ID NO: 2884 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2884 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 84.2**.

Table 84.2. Activity relative to SEQ ID NO: 2884		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2884)**	**FIOP activity relative to SEQ ID NO: 2884**
2925/2926	D183V	++
2927/2928	A408D	++
2929/2930	K397Q	++
2931/2932	G413T	++
2933/2934	A252H	+
2935/2936	Y259F/R263S	+
2937/2938	M203L	+
2939/2940	V261S/R263S	+
2941/2942	T190N	+
2943/2944	D232G	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2884 and are defined as follows: "+" > 1.6, "++" > 2.0.		

Example 85

Improvements over SEQ ID NO: 2884 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0619] SEQ ID NO: 2884 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.*, saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 85.1.**

[0620] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 85.1
Reaction buffer: 100 mM TEA, 1 mM $CoCl_2$, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (μL): 0.1; NTP: mGTP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T21mA*fG*mU, 5'-6-FAM-T31mUmUmGfU, 5'-6-FAM-T36fCmAfUfC; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mU*fA*fA, T8mA*fG*mU, T8mUmUmGfU, T8fCmAfUfC; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fAmG-3'P, 5'-6-FAM-T21mA*fG*mUmG-3'P, 5'-6-FAM-T31mUmUmGfUmG-3'P, 5'-6-FAM-T36fCmAfUfCmG-3'P; Unlabeled products: T8mU*fA*fAmG-3'P, T8mA*fG*mUmG-3'P, T8mUmUmGfUmG-3'P, T8fCmAfUfCmG-3'P; IPP: SEQ ID NO: 1926; IPP (μM): 1.

[0621] Activity relative to SEQ ID NO: 2884 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2884 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 85.2**.

Table 85.2. Activity relative to SEQ ID NO: 2884		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2884)**	**FIOP activity relative to SEQ ID NO: 2884**
2927/2928	A408D	+
2945/2946	Q208G	++
2947/2948	D223L	++
2949/2950	W229S	++
2951/2952	W229G	+
2953/2954	A201R	+
2955/2956	I230V	+
2957/2958	K179V	+
2959/2960	S297Q	+
2961/2962	L227G	+
2963/2964	V218I	+
2965/2966	K235N	+
2967/2968	L227S	+
2969/2970	K200Q	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2884 and are defined as follows: "+" > 1.17, "++" > 1.27.		

Example 86

Improvements over SEQ ID NO: 2910 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0622] SEQ ID NO: 2910 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 86.1**.

[0623] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 86.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration(vol%) 6.25; Preincubation Temp (°C): 72; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: fCTP-3'P; NTP conc (µM): 4000; Labeled oligonucleotide: 5'-6-FAM-T41mAmUmCmU; Concentration labeled oligo (µM): 25; Unlabeled oligonucleotide: T8mAmUmCmU; Concentration unlabeled oligo (µM): 1975; Labeled product: 5'-6-FAM-T41mAmUmCmUfC-3'P; Unlabeled product T8mAmUmCmUfC-3'P; IPP: SEQ ID NO: 1062; IPP (µM): 1.

[0624] Activity relative to SEQ ID NO: 2910 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2910 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 86.2**.

Table 86.2. Activity relative to SEQ ID NO: 2910		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2910)**	**FIOP activity relative to SEQ ID NO: 2910**
2971/2972	T307K/R429A/M474L	+
2973/2974	K508Q	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2910 and are defined as follows: "+" > 4.9.		

Example 87

Improvements over SEQ ID NO: 2974 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0625] SEQ ID NO: 2974 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 87.1**.

[0626] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 μM oligonucleotide, 600-12000 μM nucleotide triphosphate, 0.2-1 μM IPP, 100 mM buffer, and 1000 μM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 87.1
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (μL): 0.1; NTP: fCTP-3'P; NTP conc (μM): 12000; Labeled oligonucleotide: 5'-6-FAM-T11mU*fA*fG*fA, 5'-6-FAM-T16*fA*fA*fGmA, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T31mUfCmAfU; Concentration each labeled oligo (μM): 25; Unlabeled oligonucleotide: T8mU*fA*fG*fA, T8*fA*fA*fGmA, T8mGfUfAfC, T8mUfCmAfU; Concentration each unlabeled oligo (μM): 1975; Labeled products: 5'-6-FAM-T11mU*fA*fG*fAfC-3'P, 5'-6-FAM-T16*fA*fA*fGmAfC-3'P, 5'-6-FAM-T21mGfUfAfCfC-3'P, 5'-6-FAM-T31mUfCmAfUfC-3'P; Unlabeled products: T8mU*fA*fG*fAfC-3'P, T8*fA*fA*fGmAfC-3'P, T8mGfUfAfCfC-3'P, T8mUfCmAfUfC-3'P; IPP: SEQ ID NO: 1926; IPP (μM): 1.

[0627] Activity relative to SEQ ID NO: 2974 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2974 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 87.2**.

Table 87.2. Activity relative to SEQ ID NO: 2974		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2974)**	**FIOP activity relative to SEQ ID NO: 2974**
2975/2976	L205R/L403F	++
2977/2978	L205R/G413T	++
2979/2980	L205R/E239S/L403F/Q508K	++

Table 87.2. Activity relative to SEQ ID NO: 2974		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2974)**	**FIOP activity relative to SEQ ID NO: 2974**
2981/2982	L205R/Q208G	+
2983/2984	L205R/Q508K	+
2985/2986	L205R/S297Q/Q508K	+
2987/2988	L205R/D223L	+
2989/2990	T190N/M203F/L205R/Q508K	+
2991/2992	V218I/V319M/Q508K	+
2993/2994	T190N	+
2995/2996	L205R/Q370R/L403F	+
2997/2998	L205R/I230V	+
Levels of increased activity were determined for FIOP activity relative to SEQ ID NO: 2974 and are defined as follows: "+" > 1.29, "++" > 1.37.		

Example 88

Improvements over SEQ ID NO: 2998 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

HTP Screening for Improved TnT Variants

[0628] SEQ ID NO: 2998 was selected as the parent TnT enzyme. Libraries of engineered genes were produced from the parent gene using various techniques (*e.g.,* saturation mutagenesis and recombination of previously identified beneficial mutations). The polypeptides encoded by each gene were produced in HTP and prepared as described in **Table 88.1**.

[0629] Reactions were performed in 96-well or 384-well format BioRad PCR plates. Reactions included 600-9540 µM oligonucleotide, 600-12000 µM nucleotide triphosphate, 0.2-1 µM IPP, 100 mM buffer, and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the reaction plates (ii) TnT lysate solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for CE analysis as described in Example 4.

Table 88.1 Reaction conditions
Reaction buffer: 100 mM TEA, 1 mM CoCl2, pH 7.8; Lysate concentration (vol%): 6.25; Preincubation Temp (°C): 50; Preincubation Time (min): 60; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 0.1; NTP: mATP-3'P; NTP conc (µM): 9000; Labeled oligonucleotide: 5'-6-FAM-T26fAfAmGfC, 5'-6-FAM-T16*fU*fUfAfU; Concentration each labeled oligo (µM): 25; Unlabeled oligonucleotide: T8fAfAmGfC, T8*fU*fUfAfU; Concentration each unlabeled oligo (µM): 2975; Labeled products: 5'-6-FAM-T26fAfAmGfCmA-3'P, 5'-6-FAM-T16*fU*fUfAfUmA-3'P; Unlabeled products: T8fAfAmGfCmA-3'P, T8*fU*fUfAfUmA-3'P; IPP: SEQ ID NO: 1926; IPP (µM): 1

[0630] Activity relative to SEQ ID NO: 2998 (Activity FIOP) was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area, compared with the percent product observed by the reaction with SEQ ID NO: 2998 (where the percent product may be set as the average of replicates or else the highest single sample as appropriate). The results are shown in **Table 88.2**.

Table 88.2. Activity relative to SEQ ID NO: 2998		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2998)**	**Activity relative to SEQ ID NO: 2998**
2999/3000	L403F/I466L	+
3001/3002	V230I/Q251A/P352T/V357I	+
3003/3004	M238L/S297V/L403F/I466L	+
3005/3006	M238L/S297V/L403F	+
3007/3008	S297V	+
Levels of increased activity were determined for activity FIOP relative to SEQ ID NO: 2998 and are defined as follows: "+" > 1.12.		

Example 89

Improvements over SEQ ID NO: 682 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

Activity of shake-flask purified TnT variant with short oligonucleotides.

[0631] TnT variants SEQ ID NOs: 682, 1742, and 2452 were produced in shake flask and purified as described in Example 3. The variants were evaluated for activity with a series of 3-4 bp oligonucleotides including one containing a 5' VPU modification.

[0632] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 5000 µM oligonucleotide, 7500 µM mNTP-3'P, 5 µM TnT, 1 µM IPP (SEQ ID NO: 1926), 100 mM MOPS (pH 8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for LCMS analysis as described in Example 55.

Table 89.1
Reaction buffer: 100 mM MOPS,1 mM CoCl2, pH 8; TnT concentration (µM) 5; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 5; NTP conc (µM): 7500; Concentration oligo (µM): 5000; substrates and product in **Table 89.2**; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0633] Percent conversion was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area. The results are shown in **Table 89.2**.

Table 89.2- Fold improvement in conversion to 3'-phosphorylated extension product relative to SEQ ID NO: 682.					
NTP-3'P	Oligo substrate	Product	SEQ ID NO: 682	SEQ ID NO: 1742	SEQ ID NO: 2452
fATP-3'P	mA*mA*fGmC	mA*mA*fGmCfA-3'P	1.0	1.1	1.2
fATP-3'P	mA*fC*mAfA	mA*fC*mAfAfA-3'P	1.0	1.5	1.8
fATP-3'P	mA*fC*mA	mA*fC*mAfA-3'P	1.0	8.2	16.1
mATP-3'P	mC*mU*mAmG	mC*mU*mAmGmA-3'P	1.0	1.0	1.0
mATP-3'P	mUmCmU	mUmCmUmA-3'P	1.0	2.0	2.1
mGTP-3'P	mU*fU*mAmG	mU*fU*mAmGmG-3'P	1.0	1.3	1.5
mGTP-3'P	mC*mU*mA	mC*mU*mAmG-3'P	1.0	2.3	3.5
mGTP-3'P	(VPU)*fU*mA	(VPU)*fU*mAmG-3'P	1.0	1.6	2.0
mGTP-3'P	mU*fU*mA	mU*fU*mAmG-3'P	1.0	11.2	40.4
mUTP-3'P	mUfU*mUfA	mUfU*mUfAmU-3'P	1.0	1.4	1.4

Example 90

Improvements over SEQ ID NO: 682 in the extension of oligonucleotide acceptor molecules with nucleotide triphosphate donors

Activity of shake-flask purified TnT variant with short oligonucleotides

[0634] TnT variants SEQ ID NOs: 682, 1742, 2452, and 2998 were produced in shake flask and purified as described in Example 3. The variants were evaluated for activity with a series of oligonucleotides including those containing TGN and Chd modifications.

[0635] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Reactions included 5000 µM oligonucleotide, 7500 µM mNTP-3'P, 5 µM TnT, 1 µM IPP (SEQ ID NO: 1926), 100 mM MOPS (pH 8), and 1000 µM cobalt (II) chloride. The reactions were set up as follows: (i) all reaction components, except for TnT, were pre-mixed in a single solution, and were aliquoted into each well of the 96-well plates (ii) TnT solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at the indicated temperature and reaction time, then held at 4 °C until the reaction was quenched. Reactions were quenched and processed for LCMS analysis as described in Example 55.

Table 90.1
Reaction buffer: 100 mM MOPS,1 mM CoCl2, pH 8; TnT concentration (µM) 5; Reaction temperature (°C): 50; Reaction Time (min): 120; Reaction volume (µL): 5; NTP conc (µM): 7500; Concentration oligo (µM): 5000; substrates and product in **table 90.2**; IPP: SEQ ID NO: 1926; IPP (µM): 1.

[0636] Percent conversion was calculated as the percent product of the variant, defined as the sum of the area of products divided by the sum of the total peak area. The results are shown in **Table 90.2**.

Table 90.2- Fold improvement in conversion to 3'-phosphorylated extension product relative to SEQ ID NO: 682.						
Oligo Substrate	NQP	Product	SEQ ID NO: 682	SEQ ID NO: 1742	SEQ ID NO: 2452	SEQ ID NO: 2998
(VPU)*fU*mAmG	mGTP-3'P	(VPU)*fU*mAmGmG-3'P	1	2	3	3
(VPU)*fU*mAmG mGmU(Tgn)mG	mGTP-3'P	(VPU)*fU*mAmGmGmU(Tgn)mGmG-3'P	1	2	3	5
mU*fU*mAmGmG mU(Tgn)mG	mGTP-3'P	mU*fU*mAmGmGmU(Tgn)mGmG-3'P	1	3	7	10
mG*mG*mCmUm A(Chd)mG	mATP-3'P	mG*mG*mCmUmA(Chd)mGmGmA-3'P	1	18	25	112

[0637] While the invention has been described with reference to the specific embodiments, various changes can be made and equivalents can be substituted to adapt to a particular situation, material, composition of matter, process, process step or steps, thereby achieving benefits of the invention without departing from the scope of what is claimed.

[0638] For all purposes in the United States of America, each and every publication and patent document cited in this disclosure is incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute an admission as to its contents or date.

CLAIMS

What is claimed is:

1. An engineered terminal nucleotidyl transferase, or a functional fragment thereof, comprising an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of an even-numbered SEQ ID NO. of SEQ ID NOs: 2, 4-1056, 1130-1880, and 1928-3008, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 2, 4-1056, 1130-1880, and 1928-3008, wherein the amino acid sequence comprises one or more amino differences relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

2. The engineered terminal nucleotidyl transferase of Claim 1, comprising an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, wherein the amino acid sequence comprises one or more amino differences relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

3. The engineered terminal nucleotidyl transferase of Claim 1 or 2, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272,

273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, or 526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to a reference sequence corresponding to SEQ ID NO: 2, 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

4. The engineered terminal nucleotidyl transferase of Claim 1, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference or amino acid residue 22P, 27P, 34Y, 34D, 41T, 42T, 43-/T, 45T, 52G, 60H, 92C, 105G, 133E, 152H, 160A/D/T/W, 161A/G/L/Q/T, 162P, 164Q, 169L, 173H, 173G/P, 174V/W, 174L/W, 174I, 175E/S/T/V, 175H/L/P/R, 176N, 176H, 177C/K, 179V, 179C/K/L/R, 182I/V, 183G/R/S/T/V, 184K/R, 186G/L/M/R/T/V, 187L/T/V/Y, 187T, 190A/R, 190A/F/H/L/T/V, 190G/M/N/Q/R/V, 191F/W/Y, 192A/H, 193R/T, 193A/G/I/R/S/V, 193Q/S/T/W, 194L/M, 195F/Q/R/T/W, 196D/H/K/L/Q/R/S/V, 197I/L, 197M/Q/R, 198E/H/P/Q/R/S/T, 199E/F/V, 200Q, 201D/E/P/Q/R/S, 202H/M/S/V, 203M, 203E/F/L/Q, 204M/V, 205R, 205L, 208A/G, 208E/Q, 208D/E/G, 208E/G/M/T, 212R, 213G, 218I, 219D/Q/S, 221I/Q, 222N, 222A, 223H/L/T, 224L/V, 227G/S, 229F/G/L/S, 230C/L/V, 230I, 231N, 231C/M/N/T, 232E/G, 233I/P, 234T, 235N/R, 236R/S, 237L, 238F/I/L/V, 239G/L/R/S, 241L, 242L, 244G/I/R/S, 246L/V, 249L/M/Q, 250G, 251A/E/G/M/R/S/W, 252E/H/L/M/R/S, 254R, 254N/Q/R, 254I, 259F, 260R, 260K/Q/S/T, 261G/R/V, 261S/T, 262L/M, 263A/K/R/S/W, 263S, 263H/R/T, 264L, 264A/R/S, 267S, 267G, 268L, 272R, 273K/M, 273E/G/M/N/Q, 273I/V, 273L/M/V, 273R, 274P/R, 274L/N/R/S/V, 275I/L/R/S, 277S/T, 277D, 278D/R/S, 278A/L, 280W, 281A/C/G/H/L/S, 282N, 288D, 290L, 291K/L, 292A, 293R, 294G/T, 295T, 295A/D/N, 296V, 296L/M, 297A/S, 297A/G/L/P/Q/T/V/W/Y, 298I, 299G/H/N/P, 299A/G/N/P/R/S, 300S, 300A/K, 300R, 300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, 301A/F/V, 302S, 303E/R/T, 304L, 307D/E/K/M/N/S, 308L/Y, 308H/L, 309H, 310D/S, 312M, 313H/R/S/T, 313A/Q, 314E, 314A/I/S/T, 315K/R, 316L, 317R, 318P/R/S, 318I/K/L/Q, 319M/T, 319G/Q/V/W, 319A/G/L/M/P, 321R/T, 322A/E, 323T, 324A/T, 325G/S/V/Y, 328A/I, 329Q, 329H/L, 333N/S, 334S, 335V, 336C/T, 337S, 339I/T/V, 339A/S, 339T, 340L/M/T/V, 341L/T, 342S/T, 342S, 343L, 343I/M/R/T/V, 347A/S, 352A/E/L/M/N/T, 353R/V, 353K/L/T/V, 353V, 354G, 357I, 359A/C/F/V, 360V, 361L/V, 361I, 362A/M, 363G, 363P/T, 364S, 366F/H/P, 367N/Q/R/S, 367E/V, 368L/S, 368R, 370L/R, 371G/R, 376A/G/H/R/S/T/V, 376E/S, 377R, 378M, 379M, 379L/T, 380A/K/L/S/V, 381Y, 381S, 382L/M,

383L/Q, 384A/G/S/T, 385V, 387T/V, 388V, 389A/V, 390I/L/M, 390S/T, 390T, 392E/H/N/Q/R/S, 393A/G/I/L/M/S/T/Y, 394K/N/S, 395E/F/I/L/M/Q/S/T, 396T, 397G/Q/S/T, 397E/F/H/I/K/L/M/R/V/W/Y, 398L/M, 402A/D/K/M/P/S/T, 402P, 403E/F/I/P, 404A/S, 404E/G/I/L/M/Q/R/T/V, 405G/L/S/V, 405A/P/Q/T/V/Y, 406A/D/H/M/N/P/R/T, 406E/G/M/T, 407P/S, 407P, 407A/D/G/L/N/Q/S/V/Y, 408D/G/L/N/V, 408A, 409E/G/P/R/S/V/Y, 409D, 410V, 411E/H/K/R/T/V, 412M/N/P/Q/S/V, 413T, 414Q/R, 415G/P/S, 416G/S, 416A/H, 417A/G/S, 418C/S/T, 419A/I/M, 420C/L/V, 424P, 425R, 426G, 427A/M, 428A/L/T, 429A/S, 430G/Q/S/W, 431G, 432I/V, 433A/P/T/V, 434G/L/P, 435F/L/T, 436C/G/H/L/S, 437A/H/P, 438P, 439C/L/P, 440D/M, 441E/T, 442L/R, 443A/D/P/V, 444G, 446D/G/H/S/T, 448R, 450V, 451R, 456M, 457T, 458K, 459L, 460A/D/L/R/S/V, 461A/V, 462M, 462F/Y, 462H/L, 463P/S, 465G, 466L, 466I/L, 467M, 468H/I/M, 474L, 477I, 477E/L/R/S/T/W, 477Q/S, 479M/T/V, 480K/M/W, 481H, 484A/L/W/Y, 485I/L, 485E/V, 487R, 487K, 490I/V, 492M/S/Y, 492A/G, 492C/G/H/R/S, 493R, 494L/M/Q/R/T/V/Y, 495A/S, 495G/L/T, 497L, 499L/N/R, 500A/N, 501K/L, 502M, 503L/R/V, 504P, 506A/K/L, 507G, 508C/D/L/Q, 508K, 513V, 515G, 517I, 518S, 522M/V, 523A/D/H, 525H/I/R/T/V/W, 525G/T, or 526E, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

5. The engineered terminal nucleotidyl transferase of Claim 1 or 3, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference at amino acid position(s) 233/402, 296/384/412/415/451/477/480, 296/415/451/477/480, 296/451/477, 296/451/480, 384/412/451/480, 384/451/477, 402/404/460, 404, 404/431, 415/451/477/480/503, 451, 451/477, or 477, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

6. The engineered terminal nucleotidyl transferase of Claim 1 or 3, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference(s) 233P/402K, 296V/384S/412M/415P/451R/477Q/480W, 296V/415P/451R/477Q/480W, 296V/451R/477Q, 296V/451R/480W, 384A/412M/451R/480W, 384S/451R/477S, 402K/404A/460A, 402K/404A/460S, 404A, 404A/431G, 415P/451R/477Q/480W/503R, 451R, 451R/477Q, or 477Q, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2, or to a reference sequence corresponding to SEQ ID NO: 2.

7. The engineered terminal nucleotidyl transferase of Claim 1 or 3, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference at amino acid position 22, 27, 34, 41, 42, 43, 45, 52, 60, 92, 105, 133, 152, 160, 161, 162, 164, 169, 173, 174, 175, 176, 177, 179, 182, 183, 184, 186, 187, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 208, 212, 213, 218, 219, 221, 222, 223, 224, 227, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 241, 242, 244, 246, 249, 250, 251, 252, 254, 259, 260, 261, 262, 263, 264, 267, 268, 272, 273, 274, 275, 277, 278, 280, 281, 282, 288, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302,

303, 304, 307, 308, 309, 310, 312, 313, 314, 315, 316, 317, 318, 319, 321, 322, 323, 324, 325, 328, 329, 333, 334, 335, 336, 337, 339, 340, 341, 342, 343, 347, 352, 353, 354, 357, 359, 360, 361, 362, 363, 364, 366, 367, 368, 370, 371, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 446, 448, 450, 451, 456, 457, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 474, 477, 479, 480, 481, 484, 485, 487, 490, 492, 493, 494, 495, 497, 499, 500, 501, 502, 503, 504, 506, 507, 508, 513, 515, 517, 518, 522, 523, 525, or 526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

8. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises at least an amino acid residue difference or amino acid residue 22P, 27P, 34Y, 34D, 41T, 42T, 43-/T, 45T, 52G, 60H, 92C, 105G, 133E, 152H, 160A/D/T/W, 161A/G/L/Q/T, 162P, 164Q, 169L, 173H, 173G/P, 174V/W, 174L/W, 174I, 175E/S/T/V, 175H/L/P/R, 176N, 176H, 177C/K, 179V, 179C/K/L/R, 182I/V, 183G/R/S/T/V, 184K/R, 186G/L/M/R/T/V, 187L/T/V/Y, 187T, 190A/R, 190A/F/H/L/T/V, 190G/M/N/Q/R/V, 191F/W/Y, 192A/H, 193R/T, 193A/G/I/R/S/V, 193Q/S/T/W, 194L/M, 195F/Q/R/T/W, 196D/H/K/L/Q/R/S/V, 197I/L, 197M/Q/R, 198E/H/P/Q/R/S/T, 199E/F/V, 200Q, 201D/E/P/Q/R/S, 202H/M/S/V, 203M, 203E/F/L/Q, 204M/V, 205R, 205L, 208A/G, 208E/Q, 208D/E/G, 208E/G/M/T, 212R, 213G, 218I, 219D/Q/S, 221I/Q, 222N, 222A, 223H/L/T, 224L/V, 227G/S, 229F/G/L/S, 230C/L/V, 230I, 231N, 231C/M/N/T, 232E/G, 233I/P, 234T, 235N/R, 236R/S, 237L, 238F/I/L/V, 239G/L/R/S, 241L, 242L, 244G/I/R/S, 246L/V, 249L/M/Q, 250G, 251A/E/G/M/R/S/W, 252E/H/L/M/R/S, 254R, 254N/Q/R, 254I, 259F, 260R, 260K/Q/S/T, 261G/R/V, 261S/T, 262L/M, 263A/K/R/S/W, 263S, 263H/R/T, 264L, 264A/R/S, 267S, 267G, 268L, 272R, 273K/M, 273E/G/M/N/Q, 273I/V, 273L/M/V, 273R, 274P/R, 274L/N/R/S/V, 275I/L/R/S, 277S/T, 277D, 278D/R/S, 278A/L, 280W, 281A/C/G/H/L/S, 282N, 288D, 290L, 291K/L, 292A, 293R, 294G/T, 295T, 295A/D/N, 296V, 296L/M, 297A/S, 297A/G/L/P/Q/T/V/W/Y, 298I, 299G/H/N/P, 299A/G/N/P/R/S, 300S, 300A/K, 300R, 300A/E/F/G/I/K/L/M/N/Q/R/S/V/Y, 301A/F/V, 302S, 303E/R/T, 304L, 307D/E/K/M/N/S, 308L/Y, 308H/L, 309H, 310D/S, 312M, 313H/R/S/T, 313A/Q, 314E, 314A/I/S/T, 315K/R, 316L, 317R, 318P/R/S, 318I/K/L/Q, 319M/T, 319G/Q/V/W, 319A/G/L/M/P, 321R/T, 322A/E, 323T, 324A/T, 325G/S/V/Y, 328A/I, 329Q, 329H/L, 333N/S, 334S, 335V, 336C/T, 337S, 339I/T/V, 339A/S, 339T, 340L/M/T/V, 341L/T, 342S/T, 342S, 343L, 343I/M/R/T/V, 347A/S, 352A/E/L/M/N/T, 353R/V, 353K/L/T/V, 353V, 354G, 357I, 359A/C/F/V, 360V, 361L/V, 361I, 362A/M, 363G, 363P/T, 364S, 366F/H/P, 367N/Q/R/S, 367E/V, 368L/S, 368R, 370L/R, 371G/R, 376A/G/H/R/S/T/V, 376E/S, 377R, 378M, 379M, 379L/T, 380A/K/L/S/V, 381Y, 381S, 382L/M, 383L/Q, 384A/G/S/T, 385V, 387T/V, 388V, 389A/V, 390I/L/M, 390S/T, 390T,

392E/H/N/Q/R/S, 393A/G/I/L/M/S/T/Y, 394K/N/S, 395E/F/I/L/M/Q/S/T, 396T, 397G/Q/S/T, 397E/F/H/I/K/L/M/R/V/W/Y, 398L/M, 402A/D/K/M/P/S/T, 402P, 403E/F/I/P, 404A/S, 404E/G/I/L/M/Q/R/T/V, 405G/L/S/V, 405A/P/Q/T/V/Y, 406A/D/H/M/N/P/R/T, 406E/G/M/T, 407P/S, 407P, 407A/D/G/L/N/Q/S/V/Y, 408D/G/L/N/V, 408A, 409E/G/P/R/S/V/Y, 409D, 410V, 411E/H/K/R/T/V, 412M/N/P/Q/S/V, 413T, 414Q/R, 415G/P/S, 416G/S, 416A/H, 417A/G/S, 418C/S/T, 419A/I/M, 420C/L/V, 424P, 425R, 426G, 427A/M, 428A/L/T, 429A/S, 430G/Q/S/W, 431G, 432I/V, 433A/P/T/V, 434G/L/P, 435F/L/T, 436C/G/H/L/S, 437A/H/P, 438P, 439C/L/P, 440D/M, 441E/T, 442L/R, 443A/D/P/V, 444G, 446D/G/H/S/T, 448R, 450V, 451R, 456M, 457T, 458K, 459L, 460A/D/L/R/S/V, 461A/V, 462M, 462F/Y, 462H/L, 463P/S, 465G, 466L, 466I/L, 467M, 468H/I/M, 474L, 477I, 477E/L/R/S/T/W, 477Q/S, 479M/T/V, 480K/M/W, 481H, 484A/L/W/Y, 485I/L, 485E/V, 487R, 487K, 490I/V, 492M/S/Y, 492A/G, 492C/G/H/R/S, 493R, 494L/M/Q/R/T/V/Y, 495A/S, 495G/L/T, 497L, 499L/N/R, 500A/N, 501K/L, 502M, 503L/R/V, 504P, 506A/K/L, 507G, 508C/D/L/Q, 508K, 513V, 515G, 517I, 518S, 522M/V, 523A/D/H, 525H/I/R/T/V/W, 525G/T, or 526E, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002, or to the reference sequence corresponding to SEQ ID NO: 8, 70, 148, 184, 234, 256, 478, 668, 682, 730, 734, 742, 932, 992, 1144, 1212, 1236, 1264, 1414, 1504, 1522, 1702, 1742, 1928, 1944, 1946, 1984, 2064, 2104, 2210, 2256, 2280, 2342, 2438, 2452, 2562, 2576, 2562, 2714, 2876, 2884, 2910, 2974, 2998, or 3002.

9. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at amino acid position(s) 92/273/277/462, 267/313/314, 267/313/314/408/485, 267/313/367/485, 267/313/406, 267/314/408/441, 267/367/485, 267/406/408/485, 273/277, 273/277/381, 273/277/381/404/462/492, 273/277/381/429, 273/277/381/462, 273/277/381/462/492, 273/277/381/492, 273/277/429, 273/277/429/492, 273/277/462, 273/381, 273/381/492, 273/462, 273/462/492, 277/381, 277/381/429/462/492, 277/381/429/492, 277/381/462/492, 277/381/492, 277/429/462/492, 277/429/492, 277/492, 309/313/314/412/485, 381/462/492, or 381/492, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, or to the reference sequence corresponding to SEQ ID NO: 8; or

(b) at least an amino acid residue difference(s) 92C/273R/277T/462M, 267S/313S/314A/408V/485L, 267S/313S/367S/485L, 267S/313S/406H, 267S/313T/314A, 267S/314A/408V/441E, 267S/367S/485L, 267S/406H/408V/485L, 273R/277S, 273R/277S/381Y, 273R/277S/381Y/404S/462M/492M, 273R/277S/381Y/429A, 273R/277S/381Y/462M, 273R/277S/381Y/462M/492M, 273R/277S/381Y/462M/492Y, 273R/277S/429A, 273R/277S/429A/492S, 273R/277S/462M, 273R/277T, 273R/277T/381Y, 273R/277T/381Y/429A, 273R/277T/381Y/492M, 273R/277T/429A, 273R/277T/462M, 273R/381Y, 273R/381Y/492M, 273R/462M, 273R/462M/492S, 273R/462M/492Y, 277S/381Y, 277S/381Y/429A/462M/492M, 277S/381Y/429A/492M, 277S/381Y/462M/492M, 277S/381Y/462M/492S, 277S/429A/462M/492S, 277S/429A/492Y, 277S/492S, 277T/381Y, 277T/381Y/429A/462M/492M,

277T/381Y/492M, 277T/381Y/492S, 309H/313S/314A/412N/485L, 381Y/462M/492Y, or 381Y/492S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, or to the reference sequence corresponding to SEQ ID NO: 8; or

(c) at least an amino acid residue difference at an amino acid positions(s) 186, 193, 196, 198, 198/515, 274, 394, or 409, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, or to the reference sequence corresponding to SEQ ID NO: 8; or

(d) at least an amino acid residue difference 186R, 193Q, 193T, 193W, 196K, 198H, 198Q, 198R, 198R/515G, 198S, 274R, 394S, or 409R, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 8, or to the reference sequence corresponding to SEQ ID NO: 8.

10. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 190, 190/208/267/274, 190/208/267/274/309/417, 190/208/267/274/417/462, 190/208/274/309/313/417/462, 190/208/309/462, 190/267/274, 190/274/417, 190/274/417/477, 208/274/309/313/430, 267/274/477, 273, 274, 274/309, 274/309/313/417/462, 274/309/417/477, 274/313, or 274/462, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 70, or to the reference sequence corresponding to SEQ ID NO: 70; or

(b) at least an amino acid residue difference(s) 190A, 190A/208E/267S/274P, 190A/208E/309H/462H, 190A/208G/267S/274P/417A/462H, 190A/274P/417A, 190R/208G/267S/274P/309H/417A, 190R/208G/274P/309H/313S/417A/462H, 190R/267S/274P, 190R/274P/417A/477R, 208E/274P/309H/313S/430Q, 267S/274P/477R, 273V, 274P, 274P/309H, 274P/309H/313S/417A/462H, 274P/309H/417A/477R, 274P/313S, or 274P/462H, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 70, or to the reference sequence corresponding to SEQ ID NO: 70.

11. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 183/208/309/313/407/462, 208/343/407, 208/407, or 309, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148; or

(b) at least an amino acid residue difference(s) 183R/208E/309H/313S/407P/462H, 208D/343L/407P, 208E/343L/407P, 208E/407P, or 309H, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148; or

(c) at least an amino acid residue difference at an amino acid positions(s) 190, 268, 275, 303, 397, 409, 415, 417, and 477, or combinations thereof, wherein the amino acid positions are relative to the reference

sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148; or

(d) at least an amino acid residue difference(s) 190H, 268L, 275L, 275S, 303T, 397V, 409P, 415G, 415P, 417S, and 477R, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148; or

(e) at least an amino acid residue difference at an amino acid positions(s) 186, 304, 318, 347, 353, 409, 416, 417, and 420, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148; or

(f) at least an amino acid residue difference(s) 186L, 186T, 186V, 304L, 318L, 347A, 353L, 409P, 416H, 417S, and 420L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 148, or to the reference sequence corresponding to SEQ ID NO: 148.

12. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 193/274/411/465/525, 193/408/465, 193/465, 274/352/353/465, 281/411/465, 343/408/465, 343/411, 411, 411/525, and 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 184, or to the reference sequence corresponding to SEQ ID NO: 184; or

(b) at least an amino acid residue difference(s) 193S/274N/411T/465G/525H, 193S/408L/465G, 193S/465G, 274N/352M/353T/465G, 281S/411T/465G, 343I/408L/465G, 343I/411T, 411T, 411T/525H, and 525H, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 184, or to the reference sequence corresponding to SEQ ID NO: 184.

13. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 275, 299, 300, 392, 393, or 397, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(b) at least an amino acid residue difference(s) 275I, 299G, 299P, 299R, 299S, 300F, 300G, 300I, 300K, 300L, 300M, 300N, 300Q, 300R, 300Y, 392H, 392N, 393G, 393Y, 397F, 397H, 397I, 397K, 397M, 397R, 397V, 397W, or 397Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(c) at least an amino acid residue difference at an amino acid positions(s) 299, 300, 392, 393, 395, or 397, or combinations thereof, wherein the amino acid positions are relative to the reference sequence

corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(d) at least an amino acid residue difference(s) 299A, 299G, 299N, 299R, 299S, 300A, 300E, 300F, 300L, 300M, 300N, 300S, 300V, 300Y, 392E, 392N, 392Q, 392S, 393A, 393I, 393L, 393M, 393S, 393T, 395F, 395I, 395L, 395M, 395Q, 395T, 397E, 397L, or 397M, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(e) at least an amino acid residue difference at an amino acid positions(s) 307, 359, 405, 420, 492, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234 or

(f) at least an amino acid residue difference(s) 307S, 359F, 405T, 420V, 492H, or 525T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(g) at least an amino acid residue difference at an amino acid positions(s) 298, 308, 312, 318, 319, 322, 328, 359, 361, 379, 382, 389, 390, 392, 394, 398, 404, 405, 409, 410, 411, 412, 414, 415, 417, 419, 420, 457, 458, 466, 468, 492, 493, 494, 495, 513, 517, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234; or

(h) at least an amino acid residue difference(s) 298I, 308L, 312M, 318I, 319M, 319T, 322A, 328I, 359V, 361L, 379T, 382M, 389A, 390I, 392R, 394K, 398M, 404I, 404L, 404R, 405Y, 409G, 409R, 409S, 409Y, 410V, 411H, 411R, 411V, 412V, 414R, 415S, 417G, 419I, 419M, 420C, 457T, 458K, 466L, 468H, 468I, 468M, 492C, 492G, 492H, 492R, 492S, 493R, 494Q, 495G, 495L, 495T, 513V, 517I, or 525I, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 234, or to the reference sequence corresponding to SEQ ID NO: 234.

14. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 307, 319/410/495, 319/466/492/495, 319/492/495, 325/359/404/409, 359/404/405, 405, 405/409, 410, 412, 415, 419, 442/457, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256; or

(b) at least an amino acid residue difference(s) 307S, 319T/410V/495G, 319T/466L/492H/495G, 319T/492H/495G, 325G/359F/404T/409R, 359F/404R/405T, 405T, 405T/409R, 410V, 412V, 415P, 419M, 442R/457T, or 525T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256;

(c) at least an amino acid residue difference at an amino acid positions(s) 323, 336, 339, 362, 363, 363/434, 368, 380, 383, 384, 402, 403, 426, 428, 430, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 459, 460, 497, 499, 501, 504, or 506, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256; or

(d) at least an amino acid residue difference(s) 323T, 336C, 336T, 339T, 362M, 363P, 363P/434P, 368S, 380A, 380L, 380S, 380V, 383L, 384G, 384T, 402D, 402S, 402T, 403I, 426G, 428A, 430S, 432I, 432V, 433T, 434G, 434L, 434P, 435F, 435L, 435T, 436C, 436H, 436L, 437A, 437P, 438P, 439C, 439L, 439P, 440M, 441T, 442L, 443A, 443P, 443V, 459L, 460R, 460S, 460V, 497L, 499R, 501L, 504P, 506A, or 506L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256; or

(e) at least an amino acid residue difference at an amino acid positions(s) 329, 336, 339, 362, 363, 363/434, 368, 380, 381, 383, 384, 385, 397, 402, 425, 427, 428, 430, 432, 433, 435, 436, 437, 438, 439, 443, 459, 460, 497, 499, 500, 501, 506, 508, or 518, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256; or

(f) at least an amino acid residue difference(s) 329H, 336C, 339I, 339T, 362A, 363P/434P, 363T, 368L, 368S, 380A, 380L, 380V, 381S, 383Q, 384T, 385V, 397T, 402A, 402D, 402M, 402P, 402S, 402T, 425R, 427A, 428T, 430W, 432I, 433V, 435T, 436C, 436G, 437A, 438P, 439P, 443V, 459L, 460L, 460S, 460V, 497L, 499L, 500A, 501L, 506A, 506L, 508L, or 518S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 256, or to the reference sequence corresponding to SEQ ID NO: 256.

15. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 299/302/363/402/405/416, 299/363/402, 299/363/405/416, 299/363/459, 299/402/416/501, 299/459, 302/363/402/405, 302/402/459, 402/416/459, or 459, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 478, or to the reference sequence corresponding to SEQ ID NO: 478; or

(b) at least an amino acid residue difference(s) 299S/302S/363P/402S/405T/416A, 299S/363P/402S, 299S/363P/405T/416A, 299S/363P/459L, 299S/402S/416A/501L, 299S/459L, 302S/363P/402S/405T, 302S/402S/459L, 402S/416A/459L, or 459L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 478, or to the reference sequence corresponding to SEQ ID NO: 478.

16. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 302/416/497, 368, 368/416/435/436/501, or 416, or combinations thereof, wherein the amino acid positions are relative to the

reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668; or

(b) at least an amino acid residue difference(s) 302S/416A/497L, 368S, 368S/416A/435L/436H/501L, or 416A, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668; or

(c) at least an amino acid residue difference at an amino acid positions(s) 339/368/436, and 416, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668; or

(d) at least an amino acid residue difference(s) 339T/368S/436L, or 416A, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668; or

(e) at least an amino acid residue difference at an amino acid positions(s) 184, 191, 222, 224, 232, 236, 246, 251, 288, 291, 319, 444, 485, or 490, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668; or

(f) at least an amino acid residue difference(s) 184R, 191F, 191W, 191Y, 222A, 224L, 232E, 236S, 246V, 251R, 288D, 291K, 319G, 319W, 444G, 485I, or 490I, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 668, or to the reference sequence corresponding to SEQ ID NO: 668.

17. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions 191, 368, 485, or 490, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 682, or to the reference sequence corresponding to SEQ ID NO: 682; or

(b) at least an amino acid residue difference 191F, 368S, 485I, or 490I, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 682, or to the reference sequence corresponding to SEQ ID NO: 682.

18. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 191/222/236/246/291/368/444, 191/222/244/246/291/368/490, 191/222/244/246/368/490, 191/222/244/246/368/495, 191/222/244/291/368/444, 191/222/246/368/444, 191/222/291/368, 191/222/291/368/490, 191/222/368, 191/222/368/444/490, 191/222/368/490, 191/236/244/291/368/490, 191/236/291/368, 191/236/291/368/490, 191/246/291/368/490, 191/246/368/490, 191/291/368/444,

191/291/368/444/490, 191/368/444, 191/368/444/490, 191/368/490, 222/236/246/291/368/444/490, 222/244/291/368/444, 222/246/368/444, 222/246/490, 222/291/368, 222/291/368/444, 222/291/368/444/490, 222/368/444/490, 222/368/490, 236/246/291/368/444/490, 236/246/368, 244/246/291/368, 244/246/368/444, 244/246/368/490, 244/291/368/490, 246/291/296/368/444/490, 246/291/368, 246/291/368/490, 246/368/444, 368, 368/444, or 368/490, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730; or

(b) at least an amino acid residue difference(s) 191F/222A/236S/246V/291K/368S/444G, 191F/222A/244R/246V/291K/368S/490I, 191F/222A/244R/246V/368S/490I, 191F/222A/244R/246V/368S/495S, 191F/222A/244R/291K/368S/444G, 191F/222A/246V/368S/444G, 191F/222A/291K/368S, 191F/222A/291K/368S/490V, 191F/222A/368S, 191F/222A/368S/444G/490V, 191F/222A/368S/490I, 191F/236S/244R/291K/368S/490V, 191F/236S/291K/368S, 191F/236S/291K/368S/490V, 191F/246V/291K/368S/490I, 191F/246V/368S/490V, 191F/291K/368S/444G, 191F/291K/368S/444G/490I, 191F/368S/444G, 191F/368S/444G/490V, 191F/368S/490I, 191F/368S/490V, 222A/236S/246V/291K/368S/444G/490V, 222A/244R/291K/368S/444G, 222A/246V/368S/444G, 222A/246V/490I, 222A/291K/368S, 222A/291K/368S/444G, 222A/291K/368S/444G/490V, 222A/368S/444G/490I, 222A/368S/444G/490V, 222A/368S/490V, 236S/246V/291K/368S/444G/490V, 236S/246V/368S, 244R/246V/291K/368S, 244R/246V/368S/444G, 244R/246V/368S/490V, 244R/291K/368S/490V, 246V/291K/296M/368S/444G/490V, 246V/291K/368S, 246V/291K/368S/490V, 246V/368S/444G, 368S, 368S/444G, or 368S/490V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730; or

(c) at least an amino acid residue difference at an amino acid positions(s) 161, 173, 197, 201, 208, 262, 343, 379, 382, 416, 429, 494, 508, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730; or

(d) at least an amino acid residue difference(s) 161G, 161T, 173G, 197M, 201E, 208A, 262M, 343V, 379L, 382L, 416G, 429S, 494R, 508D, or 525W, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730; or

(e) at least an amino acid residue difference at an amino acid positions(s) 161, 164, 201, 213, 223, 263, 333, 337, 411, 429, 463, 467, 479/485, 484/485, 502, 507, 522, 523, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730; or

(f) at least an amino acid residue difference(s) 161G, 161Q, 164Q, 201E, 213G, 223T, 263A, 333N, 333S, 337S, 411E, 429S, 463P, 467M, 479V/485V, 484W/485E, 502M, 507G, 522M, 522V, 523D, or 525R, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding

to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 730, or to the reference sequence corresponding to SEQ ID NO: 730.

19. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 161, 161/197/208/262/416, 161/208, 161/208/262/411/416/429, 161/208/262/411/416/467, 161/208/467, 161/262, 161/262/411, 161/262/411/416, 161/262/411/416/467, 161/262/411/467, 161/262/416, 161/262/416/467, 161/262/429, 161/262/467, 161/416/442, 161/429, 197, 197/208, 197/208/262, 197/208/262/411/429/467, 197/208/411, 197/208/411/416/467, 197/208/416, 197/208/467, 197/262, 197/262/467, 208, 208/262, 208/262/411/416, 208/262/416/467, 208/262/429, 208/411/416, 208/411/467, 208/416, 208/416/429, 262, 262/411, 262/411/416, 262/411/416/429, 262/416, 262/416/467, 411/416, or 416, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 742, or to the reference sequence corresponding to SEQ ID NO: 742; or

(b) at least an amino acid residue difference(s) 161G, 161G/197M/208G/262M/416G, 161G/208G, 161G/208G/262M/411E/416G/429S, 161G/208G/262M/411E/416G/467M, 161G/208G/467M, 161G/262L/429S, 161G/262M, 161G/262M/411E, 161G/262M/411E/416G, 161G/262M/411E/416G/467M, 161G/262M/411E/467M, 161G/262M/416G, 161G/262M/416G/467M, 161G/262M/467M, 161G/416G/442R, 161G/429S, 197M, 197M/208G, 197M/208G/262M, 197M/208G/262M/411E/429S/467M, 197M/208G/411E, 197M/208G/411E/416G/467M, 197M/208G/416G, 197M/208G/467M, 197M/262M, 197M/262M/467M, 208G, 208G/262M, 208G/262M/411E/416G, 208G/262M/416G/467M, 208G/262M/429S, 208G/411E/416G, 208G/411E/467M, 208G/416G, 208G/416G/429S, 262M, 262M/411E, 262M/411E/416G, 262M/411E/416G/429S, 262M/416G, 262M/416G/467M, 411E/416G, or 416G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 742, or to the reference sequence corresponding to SEQ ID NO: 742.

20. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 262/317, 262/329, 262/339, 262/403, 262/462, 262/492, 262/526, or 387, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 932, or to the reference sequence corresponding to SEQ ID NO: 932; or

(b) at least an amino acid residue difference(s) 262M/317R, 262M/329L, 262M/339V, 262M/403E, 262M/403F, 262M/403P, 262M/462L, 262M/492A, 262M/526E, or 387V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 932, or to the reference sequence corresponding to SEQ ID NO: 932; or

(c) at least an amino acid residue difference at an amino acid positions(s) 184/262, 262/340, 262/363, 262/371, 262/371/443, 262/377, or 262/387, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 932, or to the reference sequence corresponding to SEQ ID NO: 932; or

(d) at least an amino acid residue difference(s) 184K/262M, 262M/340V, 262M/363G, 262M/371G/443D, 262M/371R, 262M/377R, or 262M/387T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 932, or to the reference sequence corresponding to SEQ ID NO: 932.

21. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 183, 186, 193, 264, 294, 297, 313, 314, 315, 347, 352, 353, 359, 389, 398, 405, 406, and 409, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 734, or to the reference sequence corresponding to SEQ ID NO: 734; or

(b) at least an amino acid residue difference(s) 183R, 183S, 186G, 186L, 186M, 186T, 186V, 193R, 264L, 294G, 297A, 297S, 313H, 313S, 314A, 314S, 315K, 315R, 347S, 352A, 352N, 353K, 359A, 359C, 389V, 398L, 405P, 406R, and 409P, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 734, or to the reference sequence corresponding to SEQ ID NO: 734.

22. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 60/193/264/297, 60/263/264/297/300/525, 193/297, 208/273/294/407, 208/273/295, 273, and 297, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 992, or to the reference sequence corresponding to SEQ ID NO: 992; or

(b) at least an amino acid residue difference(s) 60H/193T/264L/297S, 60H/263A/264L/297S/300S/525W, 193T/297A, 208E/273L/294T/407G, 208E/273M/295N, 273L, or 297S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 992, or to the reference sequence corresponding to SEQ ID NO: 992.

23. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

at least an amino acid residue difference at an amino acid positions(s) 229, 230, 231, 233, 234, 237, 238, 241, 250, 251, 252, 260/264, or 264, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1144, or to the reference sequence corresponding to SEQ ID NO: 1144; or

(b) at least an amino acid residue difference(s) 229F, 230C, 230L, 231C, 231M, 231T, 233I, 234T, 237L, 238F, 238L, 238V, 241L, 250G, 251E, 251G, 251M, 251R, 251S, 251W, 252M, 252S, 260R/264A, and 264A, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1144, or to the reference sequence corresponding to SEQ ID NO: 1144; or

(c) at least an amino acid residue difference at an amino acid positions(s) 329, 329/339/340, 329/339/340/371/387, 329/339/340/403, 329/339/340/403/526, 329/339/371/387, 329/339/403/443, 329/526, 339, 339/340/387/526, 339/340/428, 339/403, 339/526, 371/403/526, and 443/526, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1144, or to the reference sequence corresponding to SEQ ID NO: 1144; or

(d) least an amino acid residue difference(s) 329L, 329L/339V/340V, 329L/339V/340V/371G/387T, 329L/339V/340V/403E, 329L/339V/340V/403E/526E, 329L/339V/371G/387T, 329L/339V/403E/443D, 329L/526E, 339V, 339V/340V/387V/526E, 339V/340V/428L, 339V/403E, 339V/526E, 371G/403E/526E, and 443D/526E, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1144, or to the reference sequence corresponding to SEQ ID NO: 1144.

24. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 184/329/403/404/492, 184/403/404/526, 329, 329/526, 340, 340/460, 376, and 492, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1212, or to the reference sequence corresponding to SEQ ID NO: 1212; or

(b) at least an amino acid residue difference(s) 184K/329L/403E/404G/492A, 184K/403F/404G/526E, 329L, 329L/526E, 340M, 340V/460V, 376S, and 492A, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1212, or to the reference sequence corresponding to SEQ ID NO: 1212.

25. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 174, 190, 198, 201, 202, 212, 263, 297, 318, 319, 322/329, 325/329, 329, 343, 379, 412, 415, 419, 448, 450, 456, 463, 477, 479, 480, 484, 523, and 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1236, or to the reference sequence corresponding to SEQ ID NO: 1236; or

(b) at least an amino acid residue difference(s) 174W, 190A, 190F, 190L, 190T, 190V, 198E, 201D, 202M, 202S, 212R, 263A, 263S, 263W, 297G, 297P, 318Q, 319Q, 322E/329Q, 325G/329Q, 325S/329Q, 325Y/329Q, 329Q, 343R, 343T, 343V, 379L, 412Q, 415G, 419A, 448R, 450V, 456M, 463S, 477T, 479V, 480M, 484A, 523D, 525I, 525R, and 525V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1236, or to the reference sequence corresponding to SEQ ID NO: 1236; or

(c) at least an amino acid residue difference at an amino acid positions(s) 175, 179, 183, 192, 194, 196, 199, 203, 208, 264, 280, 281, 295, 300, 313, 314, 352, 353, 354, 389, 404, 406, 407, 408, and 409, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to

amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1236, or to the reference sequence corresponding to SEQ ID NO: 1236; or

(d) at least an amino acid residue difference(s) 175V, 179L, 183V, 192A, 194L, 196H, 199F, 203M, 208Q, 264R, 280W, 281C, 281G, 281H, 281L, 281S, 295A, 300L, 300N, 313H, 313R, 314T, 352E, 352L, 353L, 353V, 354G, 389V, 404E, 404L, 404Q, 404V, 406N, 406T, 407L, 407V, 408L, and 409E, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1236, or to the reference sequence corresponding to SEQ ID NO: 1236.

26. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 190/263/295/300/342/393, 190/300/525, 263/295/300/420, 263/295/420, 264/300/420, 264/379, 264/420, 264/420/525, 295/379/420, 295/420, 300/379/420, 300/420, 313, 313/379/525, 342, 342/420, 379, 379/420, 420, and 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264; or

(b) at least an amino acid residue difference(s) 190G/263S/295A/300S/342S/393T, 190G/300S/525V, 263S/295A/300S/420V, 263S/295A/420V, 264S/300S/420V, 264S/379L, 264S/420V, 264S/420V/525V, 295A/379L/420V, 295A/420V, 300S/379L/420V, 300S/420V, 313R, 313R/379L/525V, 342S, 342S/420V, 379L, 379L/420V, 420V, and 525V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264;

(c) at least an amino acid residue difference at an amino acid positions(s) 174, 190, 190/193, 190/194, 260, 353, 392, 393, 395, 405, 407, 408, 411, and 418, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264; or

(d) at least an amino acid residue difference(s) 174V, 190M, 190R/193I, 190R/194M, 260R, 353L, 353V, 392Q, 393T, 395E, 395M, 395T, 405Q, 407Q, 408N, 411K, and 418C, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264; or

(e) at least an amino acid residue difference at an amino acid positions(s) 190, 190/193, 190/194, 353, 405, 407, 408, and 409, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264; or

(f) at least an amino acid residue difference(s) 190R, 190R/193G, 190R/193S, 190R/193V, 190R/194M, 353L, 405Q, 405V, 407G, 407Y, 408V, and 409V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1264, or to the reference sequence corresponding to SEQ ID NO: 1264.

27. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 169/409, 194, 203/208/295/308/409, 203/208/295/389/409, 263/300/353, 295/308/409, 300, and 352/353, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1414, or to the reference sequence corresponding to SEQ ID NO: 1414; or

(b) at least an amino acid residue difference(s) 169L/409E, 194L, 203M/208Q/295A/308H/409E, 203M/208Q/295D/308H/409E, 203M/208Q/295D/389V/409E, 263S/300N/353V, 295A/308H/409E, 300N, and 352A/353V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1414, or to the reference sequence corresponding to SEQ ID NO: 1414.

28. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 174/260/263/353/405/408, 174/353, 174/405/407/420, 174/407/409/420, 260/353, 260/353/420, 263/352/353/405/408/420, 263/353/420, 263/420, 353/420, 405/408/409/420, 407/408/420, 407/409/420, and 420, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1504, or to the reference sequence corresponding to SEQ ID NO: 1504; or

(b) at least an amino acid residue difference(s) 174V/260R/263S/353L/405Q/408V, 174V/353V, 174V/405Q/407Y/420V, 174V/407Y/409D/420V, 260R/353V, 260R/353V/420V, 263S/352A/353V/405Q/408V/420V, 263S/353V/420V, 263S/420V, 353L/420V, 353V/420V, 405Q/408V/409D/420V, 407S/409D/420V, 407Y/408V/420V, and 420V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1504, or to the reference sequence corresponding to SEQ ID NO: 1504; or

(c) at least an amino acid residue difference at an amino acid positions(s) 133/197/203, 192, 197/203, 261, 263, 281, 281/440, 295/297, 295/301/308, 308, 308/314, 322, 328, 389, 390, 394, 404/409, 405/409, 461, and 484, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1504, or to the reference sequence corresponding to SEQ ID NO: 1504; or

(d) at least an amino acid residue difference(s) 133E/197Q/203E, 192H, 197R/203E, 261G, 261R, 261V, 263K, 263R, 281G, 281S/440D, 295T/297G, 295T/297P, 295T/301V/308Y, 308L, 308Y, 308Y/314I, 322E, 328A, 389V, 390L, 390M, 394N, 394S, 404I/409D, 404M/409D, 405A/409D, 405V/409D, 461A, and 484W, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1504, or to the reference sequence corresponding to SEQ ID NO: 1504.

29. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 52, 175, 182, 193, 195,

196, 201, or 278, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(b) at least an amino acid residue difference(s) 133E/197Q/203E, 192H, 197R/203E, 261G, 261R, 261V, 263K, 263R, 281G, 281S/440D, 295T/297G, 295T/297P, 295T/301V/308Y, 308L, 308Y, 308Y/314I, 322E, 328A, 389V, 390L, 390M, 394N, 394S, 404I/409D, 404M/409D, 405A/409D, 405V/409D, 461A, or 484W, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(c) at least an amino acid residue difference at an amino acid positions(s) 52, 175, 182, 193, 195, 196, 201, or 278, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(d) at least an amino acid residue difference(s) 52G, 175E, 175S, 175T, 182V, 193A, 193G, 193V, 195R, 195T, 196V, 201S, or 278R, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(e) at least an amino acid residue difference at an amino acid positions(s) 173, 193, 195, 196, 201, 272, 275, 278, 282, 297, 299, 301, 314, 412, 418, 461, 462, 479, or 494, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(f) at least an amino acid residue difference(s) 173P, 193R, 195F, 195W, 196R, 201P, 201R, 272R, 275R, 275S, 278R, 282N, 297A, 297P, 297T, 297V, 297W, 299G, 299H, 299P, 301A, 314A, 412S, 412V, 418S, 418T, 461V, 462F, 479M, 479T, 494L, 494M, 494Q, or 494V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(g) at least an amino acid residue difference at an amino acid positions(s) 174/194/195/197/319/361/420, 174/194/197/260/263/319/353/409/420, 174/194/197/260/263/353/409/420, 194/195/197/319, 194/195/319/420, 194/195/361, 194/197, 194/197/319/361/420, 194/319, 194/319/361/420, 194/319/420, 195/319/361/420, 195/319/420, 197/420, 300/325/406, 300/353/420/525, 300/406/409, 319/420, 325/406, or 361/420, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522; or

(h) at least an amino acid residue difference(s) 174L/194L/195Q/197M/319V/361V/420V, 174L/194L/197M/260Q/263H/319V/353V/409D/420V, 174L/194L/197M/260Q/263H/353V/409D/420V, 194L/195Q/197M/319V, 194L/195Q/319V/420V, 194L/195Q/361V, 194L/197M, 194L/197M/319V/361V/420V, 194L/319V, 194L/319V/361V/420V, 194L/319V/420V, 195Q/319V/361V/420V, 195Q/319V/420V, 197M/420V, 300N/325V/406D, 300N/353V/420V/525T, 300N/406D/409D, 319V/420V, 325V/406D, or 361V/420V, or combinations thereof, wherein the amino acid

positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1522, or to the reference sequence corresponding to SEQ ID NO: 1522.

30. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 261/409, 297, 300, and 409, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1702, or to the reference sequence corresponding to SEQ ID NO: 1702; or

(b) at least an amino acid residue difference(s) 261V/409D, 297P, 300N, and 409D, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1702, or to the reference sequence corresponding to SEQ ID NO: 1702.

31. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 52/201/390, 175/193/297/300, 175/297/300, 175/297/300/462, 175/300, 175/300/314, 175/300/442, 193/195/297/300/314, 193/297/300/314/442, 195/297/300/462, 221/297/300, or 297/300, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference sequence corresponding to SEQ ID NO: 1742; or

(b) at least an amino acid residue difference(s) 52G/201S/390M, 175S/193G/297V/300N, 175S/297V/300N, 175S/297V/300N/462F, 175S/300N, 175S/300N/314A, 175S/300N/442R, 193G/195T/297V/300N/314A, 193G/297V/300N/314A/442R, 195T/297A/300N/462F, 195T/297V/300N/462F, 221Q/297A/300N, 297A/300N, or 297V/300N, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference sequence corresponding to SEQ ID NO: 1742; or

(c) at least an amino acid residue difference at an amino acid positions(s) 152/281, 182, 196, 197, 261, 281, 297, 303, 307, 319, 397, 404/409, 405/409, 406/409, 409, 463, 474, or 477, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference sequence corresponding to SEQ ID NO: 1742; or

(d) at least an amino acid residue difference(s) 152H/281S, 182V, 196D, 196L, 196S, 197I, 261T, 281S, 297T, 303R, 307N, 319P, 397S, 404E/409E, 404G/409E, 405S/409E, 406A/409E, 406P/409E, 409E, 463P, 474L, or 477S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference sequence corresponding to SEQ ID NO: 1742; or

(e) at least an amino acid residue difference at an amino acid positions(s) 152/281, 190, 197, 208, 274, 281, 297, 299, 303, 307, 308, 313, 319, 321, 360, 404/409, 406/409, 417, 460, 461, 477, 479, 492, or 495, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference

sequence corresponding to SEQ ID NO: 1742; or

(f) at least an amino acid residue difference(s) 152H/281S, 190R, 197I, 208E, 208T, 274S, 281A, 281G, 281H, 281S, 297T, 299N, 303R, 303T, 307D, 307E, 307M, 308Y, 313A, 319G, 319L, 319M, 319P, 321T, 360V, 404E/409E, 404M/409E, 404V/409E, 406A/409E, 406M/409E, 406P/409E, 417G, 460D, 461A, 477E, 479M, 479T, 492G, or 495A, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1742, or to the reference sequence corresponding to SEQ ID NO: 1742.

32. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 161/251/408, 183/522, 196/231/522, 231/416/474, 273, 273/274/325/416/522, 416, or 416/474, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1928, or to the reference sequence corresponding to SEQ ID NO: 1928; or

(b) at least an amino acid residue difference(s) 161G/251M/408A, 183T/522V, 196Q/231M/522V, 231M/416S/474L, 273M, 273M/274R/325V/416G/522V, 416S, or 416S/474L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1928, or to the reference sequence corresponding to SEQ ID NO: 1928.

33. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 231/408, or 406/463, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1944, or to the reference sequence corresponding to SEQ ID NO: 1944; or

(b) at least an amino acid residue difference(s) 231M/408A, and 406P/463P, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1944, or to the reference sequence corresponding to SEQ ID NO: 1944; or

(c) at least an amino acid residue difference at an amino acid positions(s) 231, 273, 367, 407, 424, 427, 429, 430, or 499, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1944, or to the reference sequence corresponding to SEQ ID NO: 1944; or

(d) at least an amino acid residue difference(s) 231N, 273I, 273V, 367N, 367Q, 407D, 424P, 427M, 429S, 430G, 499N, or 499R, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1944, or to the reference sequence corresponding to SEQ ID NO: 1944.

34. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 196, 196/273, 196/273/325, 196/273/325/335, 196/273/325/335/367, 196/273/430, 196/335, 196/335/367, 196/335/367/430, 196/367/479, 196/430, 273, 273/274, 273/274/367/430, 273/325/335/367, 273/325/430, 273/335/430, 273/367, 273/430, 273/430/479, 274, 274/325/335/479, 274/430, 325/335, 325/335/417, 325/335/417/430/479, 325/367, 335, 335/367, 335/367/479, 335/430, 335/479, 367, 367/430, 367/479, 417, 417/430/479, 430, and 479, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1946, or to the reference sequence corresponding to SEQ ID NO: 1946; or

(b) at least an amino acid residue difference(s) 196Q, 196Q/273I/325V, 196Q/273I/325V/335V, 196Q/273I/430G, 196Q/335V, 196Q/335V/367R/430G, 196Q/367R/479M, 196Q/430G, 196S/273I, 196S/273I/325V/335V/367Q, 196S/335V/367R, 196S/367R/479M, 273I, 273I/274R, 273I/274R/367R/430G, 273I/325V/335V/367Q, 273I/325V/430G, 273I/335V/430G, 273I/367R, 273I/430G, 273I/430G/479M, 274R, 274R/325V/335V/479M, 274R/430G, 325V/335V, 325V/335V/417S, 325V/335V/417S/430G/479M, 325V/367Q, 325V/367R, 335V, 335V/367Q, 335V/367R/479M, 335V/430G, 335V/479M, 367Q, 367Q/479M, 367R, 367R/430G, 417S, 417S/430G/479M, 430G, or 479M, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1946, or to the reference sequence corresponding to SEQ ID NO: 1946.

35. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 231, 231/273, 231/273/380/417/500, 231/273/506, 231/380/417/501, 231/500/501/506, or 231/501, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1984, or to the reference sequence corresponding to SEQ ID NO: 1984; or

(b) at least an amino acid residue difference(s) 231N, 231N/273K, 231N/273K/380K/417S/500N, 231N/273K/506K, 231N/273M, 231N/380K/417S/501K, 231N/500N/501K/506K, or 231N/501K, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1984, or to the reference sequence corresponding to SEQ ID NO: 1984; or

(c) at least an amino acid residue difference at an amino acid positions(s) 187, 190, 259, 260, 263, 273, 296, 300, 314, 343, 361, 367, 376, 384, 389, 390, 393, 395, 416, 417, 428, or 446, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 1984, or to the reference sequence corresponding to SEQ ID NO: 1984; or

(d) at least an amino acid residue difference(s) 187L, 187T, 187V, 187Y, 190Q, 190V, 259F, 260K, 260S, 260T, 263R, 273M, 296L, 300A, 300K, 314E, 343M, 343V, 361I, 367E, 376E, 384S, 389V, 390S, 390T, 393T, 395F, 395S, 416G, 417A, 428L, 446D, 446G, 446H, 446S, or 446T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues

12 to the carboxy terminus of SEQ ID NO: 1984, or to the reference sequence corresponding to SEQ ID NO: 1984.

36. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 161, 162, 174, 201, 219, 223, 244, 246, 252, 254, 318, 433, 437, 503, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2064, or to the reference sequence corresponding to SEQ ID NO: 2064; or

(b) at least an amino acid residue difference(s) 161L, 162P, 174W, 201Q, 219D, 223H, 244R, 246L, 252L, 254N, 254Q, 254R, 318K, 433A, 437H, 503V, or 525T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2064, or to the reference sequence corresponding to SEQ ID NO: 2064.

37. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 174/199/389/390, 174/231, 174/231/260/273/389/390, 174/231/263/273/343, 174/231/263/389, 174/231/389/390/446, 174/231/390, 174/259/260/263/273/361/389/390/446, 174/259/273/343/361/389, 174/263/389/390, 174/273/343/389/390/446, 187/190/231/252/273/296/300/353/417, 187/190/252/273/300/417, 187/190/273/353, 187/231/252/273/296/300/353, 187/231/273/296/300/353, 187/231/296/300/353, 187/231/300/353, 187/273/353, 190/231/273, 196/199/231/263/273/389, 199/231/260/263/361/389/446, 231, 231/259/263/273, 231/273, 259/263/273/389/390, 260/273/446, 263/273/343/446, 273, 273/353, 273/389/446, or 273/390, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2104, or to the reference sequence corresponding to SEQ ID NO: 2104; or

(b) at least an amino acid residue difference(s) 174L/199V/389V/390T, 174L/231N, 174L/231N/260K/273K/389V/390T, 174L/231N/263R/389V, 174L/231N/263T/273K/343V, 174L/231N/263T/389V, 174L/231N/389V/390T/446S, 174L/231N/390T, 174L/259F/260K/263R/273K/361I/389V/390T/446S, 174L/259F/273K/343V/361I/389V, 174L/263R/389V/390T, 174L/273K/343V/389V/390T/446S, 187V/190Q/252M/273K/300K/417S, 187V/190Q/273K/353V, 187V/231N/252E/273K/296L/300K/353R, 187V/231N/300K/353V, 187V/273K/353V, 187Y/190Q/231N/252M/273K/296L/300K/353V/417S, 187Y/231N/273K/296L/300K/353R, 187Y/231N/296L/300K/353R, 190Q/231N/273K, 196Q/199V/231N/263T/273K/389V, 199V/231N/260K/263T/361I/389V/446S, 231N, 231N/259F/263T/273K, 231N/273K, 259F/263R/273K/389V/390T, 260K/273K/446S, 263T/273K/343V/446S, 273K, 273K/353V, 273K/389V/446S, or 273K/390T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2104, or to the reference sequence corresponding to SEQ ID NO: 2104.

38. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 161/162/254/417, 161/162/318/446, 174/244/273/390/437/503, 174/244/437/525, 174/273/503, 201/376, 433, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210; or

(b) at least an amino acid residue difference(s) 161L/162P/254R/417A, 161L/162P/318K/446S, 174W/244G/437H/525T, 174W/244R/273K/390T/437H/503L, 174W/273K/503L, 201Q/376E, 433P, or 525T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210; or

(c) at least an amino acid residue difference at an amino acid positions(s) 303, 397, or 466, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210; or

(d) at least an amino acid residue difference(s) 303E, 397S, or 466I, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210; or

(e) at least an amino acid residue difference at an amino acid positions(s) 278, 290, 297, 303, 397, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210; or

(f) at least an amino acid residue difference 278S, 290L, 297L, 297Y, 303E, 397G, 397S, or 525G, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2210, or to the reference sequence corresponding to SEQ ID NO: 2210.

39. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 22, 22/162/201/244/254/318/376/417/433/437/525, 22/201/376, 22/244, 22/244/254, 22/244/433/525, 22/318/376/433/525, 22/318/376/446, 22/376/417/437/446/525, 161/162/201/244/318/376/417/437, 161/162/201/244/376, 162, 162/201/318/376/417/433/525, 162/201/318/376/433/437/446, 162/244/254/433, 162/244/318/376/417/433/437, 162/254, 162/254/318/376/417/433, 162/254/376/417/433, 162/376/525, 162/433, 201/244/318/376, 201/244/376/433/525, 201/318/376/446, 244/318/376/417, 244/318/376/417/433/437/525, 318/376, 318/376/433, 433, or 437, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2256, or to the reference sequence corresponding to SEQ ID NO: 2256; or

(b) at least an amino acid residue difference(s) 22P, 22P/162P/201Q/244G/254R/318K/376E/417A/433P/437H/525T, 22P/201Q/376E, 22P/244G, 22P/244G/254R, 22P/244G/433P/525T, 22P/318K/376E/433P/525T, 22P/318K/376E/446S, 22P/376E/417A/437H/446S/525T, 161L/162P/201Q/244G/318K/376E/417A/437H, 161L/162P/201Q/244G/376E, 162P, 162P/201Q/318K/376E/417A/433P/525T, 162P/201Q/318K/376E/433P/437H/446S, 162P/244G/254R/433P, 162P/244G/318K/376E/417A/433P/437H, 162P/254R, 162P/254R/318K/376E/417A/433P, 162P/254R/376E/417A/433P, 162P/376E/525T, 162P/433P, 201Q/244G/318K/376E, 201Q/244G/376E/433P/525T, 201Q/318K/376E/446S, 244G/318K/376E/417A, 244G/318K/376E/417A/433P/437H/525T, 318K/376E, 318K/376E/433P, 433P, or 437H, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2256, or to the reference sequence corresponding to SEQ ID NO: 2256.

40. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 22/27/437/466, 22/34/278/318/437/466, 22/466, 41/42/43/397, 43/244/397, 45/397, or 397, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280; or

(b) at least an amino acid residue difference(s) 22P/27P/437H/466I, 22P/34Y/278S/318K/437H/466I, 22P/466I, 41T/42T/43-/397S, 43T/244G/397S, 45T/397S, or 397S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280; or

(c) at least an amino acid residue difference at an amino acid positions(s) 173, 175, 176, 179, 182, 196, 197, 208, 263, 273, 274, 277, 308, 310, 319, 328, 341, 376, 388, 405, 408, 411, 462, 477, or 480, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280; or

(d) at least an amino acid residue difference(s) 173P, 175L, 175R, 176H, 179C, 179K, 179R, 182I, 196D, 197L, 208T, 263R, 263T, 273M, 274L, 277D, 308Y, 310S, 319A, 328A, 341T, 376T, 388V, 405L, 408G, 411T, 462Y, 477L, 477W, or 480K, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280; or

(e) at least an amino acid residue difference at an amino acid position 195, 203, 273, 274, 277, 278, 291, 299, 300, 324, or 477, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280; or [[****Table 70.2]]

(f) at least an amino acid residue difference 195T, 203Q, 273E, 273G, 273M, 273N, 273Q, 274L, 277D, 278D, 291L, 299G, 299H, 300S, 324T, or 477W, or combinations thereof, wherein the amino acid

positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2280, or to the reference sequence corresponding to SEQ ID NO: 2280.

41. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 34/273/300/477/480, 34/396, or 300/396/477/480, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2342, or to the reference sequence corresponding to SEQ ID NO: 234; or

(b) at least an amino acid residue difference(s) 34D/273M/300S/477L/480K, 34D/396T, or 300S/396T/477L/480K, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2342, or to the reference sequence corresponding to SEQ ID NO: 2342.

42. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 27/34/173/176/179/195/277, 27/173, 27/173/176, 27/173/176/179/273/277, 27/173/176/179/277, 27/173/179/273, 27/173/179/273/277, 27/173/179/277, 27/176/179/273, 27/179, 27/179/273/277, 27/179/277, 34, 34/173/176/197/277, 105/173/176/197/277, 173/176/179, 173/176/179/197, 173/176/179/277, 173/179, 173/179/273, 173/179/273/277, 173/277, 176/179, 176/179/197, 176/179/273, 176/179/273/277, 179, 179/197, 179/273, or 179/273/277, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2438, or to the reference sequence corresponding to SEQ ID NO: 2438; or

(b) at least an amino acid residue difference(s) 27P/34D/173P/176H/179K/195T/277D, 27P/173P, 27P/173P/176H, 27P/173P/176H/179K/273M/277D, 27P/173P/176H/179R/277D, 27P/173P/179K/273M, 27P/173P/179K/273M/277D, 27P/173P/179K/277D, 27P/176H/179R/273M, 27P/179K, 27P/179K/273M/277D, 27P/179R/277D, 34D, 34D/173P/176H/197L/277D, 105G/173P/176H/197L/277D, 173P/176H/179K/277D, 173P/176H/179R, 173P/176H/179R/197L, 173P/179K/273M/277D, 173P/179R, 173P/179R/273M, 173P/179R/273M/277D, 173P/277D, 176H/179K/273M, 176H/179K/273M/277D, 176H/179R, 176H/179R/197L, 176H/179R/273M/277D, 179K, 179K/197L, 179K/273M, 179K/273M/277D, 179R, or 179R/197L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2438, or to the reference sequence corresponding to SEQ ID NO: 2438; or

(c) at least an amino acid residue difference at an amino acid positions(s) 177, 199, 202, 229, 292, 339, 342, 352, 363, 376, 406, 407, 430, 487, 508, or 525, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2438, or to the reference sequence corresponding to SEQ ID NO: 2438; or

(d) at least an amino acid residue difference(s) 177C, 199E, 202V, 229L, 292A, 339T, 342T, 352A, 352T, 363G, 376H, 406D, 406P, 407N, 430G, 430S, 487R, 508C, or 525T, or combinations thereof, wherein

the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2438, or to the reference sequence corresponding to SEQ ID NO: 2438.

43. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 176/183, 176/183/205, 183, 183/205, 183/205/408, or 205, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2452, or to the reference sequence corresponding to SEQ ID NO: 2452; or

(b) at least an amino acid residue difference(s) 176N/183R, 176N/183R/205L, 183G, 183G/205L, 183R, 183R/205L, 183R/205L/408V, or 205L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2452, or to the reference sequence corresponding to SEQ ID NO: 2452.

44. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 177, 177/292/339/342/406, 177/292/339/406, 177/292/342/363/376/406, 177/292/342/406, 177/339/342, 177/339/342/376, 177/339/342/406, 177/339/342/406/487, 177/342/376/407, 177/342/406/430, 177/407, 177/487, 292/339/342/376/406/487, 292/342, 292/342/406, 292/406, 339/342, 339/342/407, 339/342/407/487, or 406, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2562, or to the reference sequence corresponding to SEQ ID NO: 2562; or

(b) at least an amino acid residue difference(s) 177K, 177K/292A/339T/342T/406D, 177K/292A/339T/406D, 177K/292A/339T/406P, 177K/292A/342T/363G/376H/406D, 177K/292A/342T/406P, 177K/339T/342T, 177K/339T/342T/376H, 177K/339T/342T/406D, 177K/339T/342T/406P/487R, 177K/342T/376H/407N, 177K/342T/406P/430G, 177K/407N, 177K/487R, 292A/339T/342T/376H/406P/487R, 292A/342T, 292A/342T/406D, 292A/406P, 339T/342T, 339T/342T/407N, 339T/342T/407N/487R, or 406P, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2562, or to the reference sequence corresponding to SEQ ID NO: 2562; or

(c) at least an amino acid residue difference at an amino acid positions(s) 174, 187, 190, 198, 267, 274, 277, 307, 316, 343, 359, 379, 402/407, 407, 409, 412, 414, 481, 494, or 523, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2562, or to the reference sequence corresponding to SEQ ID NO: 2562; or

(d) at least an amino acid residue difference(s) 174I, 187T, 190G, 198P, 198S, 198T, 267G, 274V, 277S, 307K, 316L, 343R, 359F, 379M, 402P/407A, 407S, 409S, 412P, 414Q, 481H, 494T, 494Y, 523A, or 523H, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2562, or to the reference sequence corresponding to SEQ ID NO: 2562.

45. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 202, 208, 221, 224, 235, 236, 238, 239, 242, 249, 251, 252, 259, 263, 405/406, 406, 477, 484/487, or 487, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(b) at least an amino acid residue difference(s) 202M, 208M, 221I, 224V, 235R, 236R, 238I, 238L, 239G, 239L, 239R, 239S, 242L, 249L, 249Q, 251A, 252R, 259F, 263R, 405G/406G, 405V/406G, 406E, 406M, 406T, 477I, 484L/487K, 484Y/487K, or 487K, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(c) at least an amino acid residue difference at an amino acid positions(s) 174/190, 174/190/202/204/254, 174/190/202/254, 174/190/204, 174/190/236, 174/202/204/254, 174/202/204/254/353, 174/204/236/254/353, 187, 187/224, 187/224/321/436, 187/224/379/436, 187/249, 187/249/321/379/436, 204/254/353, 249/379, 359/407, 359/407/412, 379, or 436, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(d) at least an amino acid residue difference(s) 174I/190G, 174I/190G/202H/204M/254I, 174I/190G/202H/254I, 174I/190G/204M, 174I/190G/236R, 174I/202H/204M/254I, 174I/202H/204M/254I/353V, 174I/204M/236R/254I/353V, 187T, 187T/224V, 187T/224V/321R/436S, 187T/224V/379M/436S, 187T/249M, 187T/249M/321R/379M/436S, 204V/254I/353V, 249M/379M, 359F/407A, 359F/407S/412P, 379M, or 436S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(e) at least an amino acid residue difference at an amino acid positions(s) 219, 293, 300, 310, 313, 318, 340, 364, or 392, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(f) at least an amino acid residue difference(s) 219Q, 293R, 300R, 310D, 313Q, 318P, 340L, 340T, 364S, 392Q, or 392S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(g) at least an amino acid residue difference at an amino acid positions(s) 160, 161, 173, 175, 222, 244, 278, 293, 300, 301, 310, 318, 319, 324, 334, 339, 341, 342, 366, 367, 368, 370, 376, or 378, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576; or

(h) at least an amino acid residue difference(s) 160A, 160D, 160T, 160W, 161A, 161L, 173H, 175H,

175P, 222N, 244I, 244S, 278A, 278L, 293R, 300R, 301F, 310D, 318P, 318R, 318S, 319P, 324A, 334S, 339A, 339S, 341L, 342S, 366F, 366H, 366P, 367V, 368R, 370L, 370R, 376A, 376G, 376R, 376S, 376V, or 378M, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2576, or to the reference sequence corresponding to SEQ ID NO: 2576.

46. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 187, 187/224, 187/224/263, 187/224/407, 187/224/407/412, 187/224/412, 187/254/263/359/379/407, 187/263, 187/359, 187/379, 224/359/407, 249/263, 249/263/407, 263, or 263/407/412, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2714, or to the reference sequence corresponding to SEQ ID NO: 2714: or

(b) at least an amino acid residue difference(s) 187T, 187T/224V, 187T/224V/263R, 187T/224V/407A, 187T/224V/407A/412P, 187T/224V/412P, 187T/254R/263R/359F/379M/407S, 187T/263R, 187T/359F, 187T/379M, 224V/359F/407A, 249M/263R, 249M/263R/407S, 263R, or 263R/407S/412P, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2714, or to the reference sequence corresponding to SEQ ID NO: 2714.

47. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 160/263/318/339, 175/244/293/364/407, 219/310, 219/310/340, 244/293/313, 244/293/334, 244/313, 244/313/334/364/407, 263/310/318/366, 263/318, 293/313/334/364, 293/334/364/407, 310/318/340, 318, or 334/364, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2876, or to the reference sequence corresponding to SEQ ID NO: 2876; or

(b) at least an amino acid residue difference(s) 60T/263R/318P/339S, 175H/244I/293R/364S/407P, 219S/310D, 219S/310D/340T, 244I/293R/313Q, 244I/313Q, 244I/313Q/334S/364S/407S, 244S/293R/334S, 263R/310D/318S/366H, 263R/318P, 293R/313Q/334S/364S, 293R/334S/364S/407P, 310D/318P/340T, 318P, or 334S/364S, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2876, or to the reference sequence corresponding to SEQ ID NO: 2876.

48. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 254/293/310, 254/293/310/334/340/407, 310/334, 334, 334/340, 334/407, 340, or 407, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884; or

(b) at least an amino acid residue difference(s) 254R/293R/310D, 254R/293R/310D/334S/340T/407P, 310D/334S, 334S, 334S/340T, 334S/407P, 340T, or 407P, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884; or

(c) at least an amino acid residue difference at an amino acid positions(s) 183, 190, 203, 232, 252, 259/263, 261/263, 397, 408, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884; or

(d) at least an amino acid residue difference(s) 183V, 190N, 203L, 232G, 252H, 259F/263S, 261S/263S, 397Q, 408D, or 413T, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884; or

at least an amino acid residue difference at an amino acid positions(s) 179, 200, 201, 208, 218, 223, 227, 229, 230, 235, 297, or 408, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884; or

(e) at least an amino acid residue difference 179V, 200Q, 201R, 208G, 218I, 223L, 227G, 227S, 229G, 229S, 230V, 235N, 297Q, or 408D, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2884, or to the reference sequence corresponding to SEQ ID NO: 2884.

49. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 307/429/474, or 508, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to SEQ ID NO: 2910; or

(b) at least an amino acid residue difference(s) 307K/429A/474L, or 508Q, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2910, or to the reference sequence corresponding to SEQ ID NO: 2910.

50. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino acid residue difference at an amino acid positions(s) 190, 190/203/205/508, 205/208, 205/223, 205/230, 205/239/403/508, 205/297/508, 205/370/403, 205/403, 205/413, 205/508, or 218/319/508, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2974, or to the reference sequence corresponding to SEQ ID NO: 2974; or

(b) at least an amino acid residue difference(s) 190N, 190N/203F/205R/508K, 205R/208G, 205R/223L, 205R/230V, 205R/239S/403F/508K, 205R/297Q/508K, 205R/370R/403F, 205R/403F,

205R/413T, 205R/508K, or 218I/319M/508K, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2974, or to the reference sequence corresponding to SEQ ID NO: 2974.

51. The engineered terminal nucleotidyl transferase of any one of Claims 1, 3, and 7, wherein the amino acid sequence of the engineered terminal nucleotidyl transferase comprises

(a) at least an amino residue difference at an amino acid position(s) 230/251/352/357, 238/297/403, 238/297/403/466, 297, or 403/466, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2998, or to the Ference sequence corresponding to SEQ ID NO: 2998; or

(b) at least an amino acid residue difference(s) 230I/251A/352T/357I, 238L/297V/403F, 238L/297V/403F/466L, 297V, or 403F/466L, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to amino acid residues 12 to the carboxy terminus of SEQ ID NO: 2998, or to the reference sequence corresponding to SEQ ID NO: 2998.

52. The engineered terminal nucleotidyl transferase of Claim 1, comprising an amino acid sequence comprising amino acid residues 12 to the carboxy terminus of an even-numbered SEQ ID NO. of SEQ ID NOs: 4-1056, 1130-1880, and 1928-3008, or an amino acid sequence comprising an even-numbered SEQ ID NO. of SEQ ID NOs: 4-1056, 1130-1880, and 1928-3008.

53. The engineered terminal nucleotidyl transferase of any of Claims 1-52, wherein the polypeptide sequence further comprises an N-terminal truncation of 1-156 amino acids.

54. The engineered terminal nucleotidyl transferase of any of Claims 1-53, wherein the engineered terminal nucleotidyl transferase polypeptide is fused with a second polypeptide; optionally, wherein the second polypeptide has inorganic pyrophosphatase (IPP) activity.

55. The engineered terminal nucleotidyl transferase of any of Claims 1-54, wherein said engineered terminal nucleotidyl transferase is capable of template-independent synthesis.

56. The engineered terminal nucleotidyl transferase of any of Claims 1-55, having at least one improved property, as compared to a wild-type or reference terminal nucleotidyl transferase or template-independent polymerase.

57. The engineered terminal nucleotidyl transferase of Claim 56, wherein said improved property is selected from increased thermostability, increased activity at elevated temperatures, increased soluble expression or isolated protein yield, decreased by-product formation, increased specific activity on one or more NTP-3'-O-RBG or natural or modified NTP substrates, increased incorporation efficiency in extension of oligo acceptor substrates, increased activity on one or more oligo acceptor substrates, and increased activity on oligo acceptor substrates of a length of three to seven nucleotides.

58. The engineered terminal nucleotidyl transferase of Claim 56, wherein said engineered terminal nucleotidyl transferase comprises increased soluble expression or isolated protein yield as compared to a wild-type or reference terminal nucleotidyl transferase or template-independent polymerase.

59. The engineered terminal nucleotidyl transferase of Claim 56, wherein said engineered terminal nucleotidyl transferase comprises increased thermal stability as compared to a wild-type or reference terminal nucleotidyl transferase or template-independent polymerase.

60. The engineered terminal nucleotidyl transferase of Claim 59, wherein said engineered terminal nucleotidyl transferase comprises increased activity after incubation at a temperature of 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 68 °C, 68.5 °C, 69 °C, 70 °C, or 72 °C as compared to a wild-type or reference terminal nucleotidyl transferase.

61. The engineered terminal nucleotidyl transferase of Claim 56, wherein said engineered terminal nucleotidyl transferase comprises increased activity on one or more NTP-3'-O-RBG or natural or modified NTP substrates or increased activity on one or more oligo acceptor substrates, or both, as compared to a wild-type or reference terminal nucleotidyl transferase or template-independent polymerase.

62. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase comprises conversion of at least one oligo acceptor substrate of 4 to 7 basepairs to the oligo acceptor substrate extended by one nucleotide with at least 1.3 fold, 1.4 fold, or 1.5 fold the activity relative to SEQ ID NO: 148 or 256.

63. The engineered terminal nucleotidyl transferase of Claim 62, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 682.

64. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase has activity in the conversion of a variety of oligo acceptor substrate of 4 to 7 basepairs to the oligo acceptor substrate extended by one nucleotide.

65. The engineered terminal nucleotidyl transferase of Claim 64, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 148, 256, or 682.

66. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase comprises at least 31% or 91% conversion an oligo acceptor substrate of 3 to 4 basepairs to the oligo acceptor substrate extended by one nucleotide.

67. The engineered terminal nucleotidyl transferase of Claim 66, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 932.

68. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase comprises at least 75% conversion of an oligo acceptor substrate to the oligo acceptor substrate extended by a glycol nucleic acid nucleotide ((S)-GNA) or a locked nucleic acid nucleotide (LNA).

69. The engineered terminal nucleotidyl transferase of Claim 68, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 992.

70. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase comprises increased substrate promiscuity for NTP-3'-O-RBG substrates of 2'-methoxyadenosine, 2'-methoxycytidine, 2'-methoxyguanosine, 2'-methoxyuridine, 2'-fluoroadenosine, 2'-fluorocytidine, 2'-fluoroguanosine, 2'-fluorouridine, adenosine ribonucleotide, cytidine ribonucleotide, guanosine ribonucleotide, uridine ribonucleotide, phosphorothioate linkage, 5'-phosphorylation modification, adenosine (S)-glycol nucleic acid, cytidine (S)-glycol nucleic acid, guanosine (S)-glycol nucleic acid, thymidine (S)-glycol nucleic acid, uridine (S)-glycol nucleic acid, C2'-endo locked adenosine ribonucleotide, C2'-endo locked cytidine ribonucleotide, C2'-endo locked guanosine ribonucleotide, C2'-endo locked thymidine ribonucleotide, C2'-endo locked uridine ribonucleotide, 2'-O-(2-methoxyethyl)adenosine, 2'-O-(2-methoxyethyl)cytidine, 2'-O-(2-methoxyethyl)guanosine, 2'-O-(2-methoxyethyl)uridine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxythymidine, 2',3'-dideoxyuridine vinylphosphonate 2'O-methoxy uridine, 1-((2S))-1-(2,3-dihydroxypropyl))thymine, and 2'O-hexadecyl cytidine, as compared to a wild-type or engineered reference TnT.

71. The engineered terminal nucleotidyl transferase of Claim 70, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence selected from the even-numbered sequences in SEQ ID NO: 4-1056, 1130-1880, and 1928-3008.

72. The engineered terminal nucleotidyl transferase of Claim 70, wherein said engineered terminal nucleotidyl transferase comprises an amino acid sequence with at least 80% sequence identity to SEQ ID NO: 992.

73. The engineered terminal nucleotidyl transferase of Claim 61, wherein said engineered terminal nucleotidyl transferase comprises increased substrate promiscuity for oligo acceptor substrates of 5'-6-FAM-T10mCmCmUfA, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T36fCmAmUmC, 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T41*fC*mAfA, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T56mA*fC*mAfA, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T51fGmUfGmU, 5'-6-FAM-T11AmC*mA*mG, 5'-6-FAM-T15mAmUmCmU, 5'-6-FAM-T46mUmCmUmU, 5'-6-FAM-T41mAmUmCmU, T8mUfGmUfC, T8fCmAmUmC, T8mCfCmCfG, T8mCfCmGfG, mAmAmAmAmUmCmU, mAmAmAmUmCmU, mAmAmUmCmU, mAmUmCmU, mUmCmU, 5'P-mUmCmU, mC*mA*mGmA, mAfGmUfG, mGmUmC*mC, 5'-6-FAM-T9mAmAmAmUmCmU, 5'-6-FAM-T9mAmAmAmUmCmU(sgna)A, 5'-6-FAM-T9mAmAmAmUmCmU+A, 5'-6-FAM-T9mAmAmAmUmCmU+G, 5'-6-FAM-T9mAmAmAmUmCmU+C, 5'-6-FAM-T9mAmAmAmUmCmU+U, 5'-6-FAM-T9mAmAmAmUmCmU+A+A, 5'-6-FAM-T9mAmAmAmUmCmU+G+G, 5'-6-FAM-T9mAmAmAmUmCmU+U+U, 5'-6-FAM-T16mAfGmAfG, 5'-6-

FAM-T26fCfAfUmG, 5'-6-FAM-T10AmC*mA*mG, 5'-6-FAM-T16*fC*mAfAfA, 5'-6-FAM-T16*fA*mAmGfAfG, 5'-6-FAM-T11fUfUfUfC, 5'-6-FAM-T21mUmAmG*mA, 5'-6-FAM-T20mAmUfUmGfG, 5'-6-FAM-T25mAmGfUfGfG, 5'-6-FAM-T20mAmGfAmGfG, 5'-6-FAM-T16*fU*fUfAfU, 5'-6-FAM-T31mUfCmAmU, 5'-6-FAM-T35fAfGfUmGfG, 5'-6-FAM-T51mAmUmCfC, 5'-6-FAM-T56fGmUfCmU, T8AmC*mA*mG, T8mCfCmGfG, T8*fC*mAfAfA, T8*fA*mAmGfAfG, T8mGfUfAfC, T8fUfUfUfC, T8mAfUfCfC, T8mAmUfUmGfG, T8mAmGfUfGfG, T8mAmGfAmGfG, T8*fU*fUfAfU, T8mGfUmGfG, T8fAmUmUfG, T8mU*fA*fAfG, T8fAfGfUmGfG, T8fGmUfCmU, and T8mA*fC*mAfA, as compared to a wild-type or engineered reference terminal nucleotidyl transferase.

74. The engineered terminal nucleotidyl transferase of any of Claims 1-73, wherein said terminal nucleotidyl transferase is purified.

75. The engineered terminal nucleotidyl transferase of any of Claims 1-74, wherein said terminal nucleotidyl transferase is immobilized.

76. A polynucleotide comprising a polynucleotide sequence encoding at least one engineered terminal nucleotidyl transferase of any of Claims 1-73.

77. The polynucleotide of Claim 76, comprising a polynucleotide sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residue 34 to the 3'-terminus of SEQ ID NO: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2713, 2875, 2883, 2909, 2973, 2997, or 3001, or to a reference polynucleotide sequence comprising SEQ ID NO: 1, 7, 69, 147, 183, 233, 255, 477, 667, 681, 729, 733, 741, 931, 991, 1143, 1211, 1235, 1263, 1413, 1503, 1521, 1701, 1741, 1927, 1943, 1945, 1983, 2063, 2103, 2209, 2255, 2279, 2341, 2437, 2451, 2561, 2575, 2713, 2875, 2883, 2909, 2973, 2997, or 3001, wherein the polynucleotide sequence encodes a terminal nucleotidyl transferase.

78. The polynucleotide of Claim 76, comprising a polynucleotide sequence having at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to the reference polynucleotide sequence corresponding to nucleotide residues 34 to 3'-terminus of an odd numbered SEQ ID NO. of SEQ ID NOs: 3-1055, 1129-1879, and 1927-3007, or to the reference polynucleotide sequence corresponding to an odd numbered SEQ ID NO. of SEQ ID NOs: 3-1055, 1129-1879, and 1927-3007.

79. The polynucleotide of any one of Claims 76-78, wherein the polynucleotide sequence is codon-optimized.

80. The polynucleotide of Claim 76, comprising a polynucleotide sequence comprising an odd numbered SEQ ID NO. of SEQ ID NOs: 3-1055, 1129-1879, and 1927-3007.

81. An expression vector comprising at least one polynucleotide of any one of Claims 76-80.

82. The polynucleotide sequence of Claims 81, wherein said polynucleotide sequence is operably linked to a control sequence.

83. A host cell comprising at least one expression vector of any one of Claims 76-82.

84. A method of producing an engineered terminal nucleotidyl transferase polypeptide in a host cell comprising culturing a host cell of Claim 83, under suitable culture conditions, such that at least one engineered terminal nucleotidyl transferase is produced.

85. The method of Claim 84, further comprising recovering at least one engineered terminal nucleotidyl transferase from the culture and/or host cells.

86. The method of Claim 84 or 85, further comprising the step of purifying said at least one engineered terminal nucleotidyl transferase.

87. A composition comprising at least one engineered terminal nucleotidyl transferase of any of Claims 1-75.

88. A method of template independent synthesis of an oligonucleotide, comprising contacting an oligonucleotide acceptor substrate with a terminal nucleotidyl transferase of any one of Claims 1-75 in presence of one or more nucleotide triphosphates under reaction conditions sufficient for addition of the nucleotide to the 3'-end of the oligonucleotide acceptor substrate.

89. The method of Claim 88, wherein the nucleotide triphosphate comprises a modified nucleotide triphosphate.

90. The method of Claim 89, wherein the modified nucleotide triphosphate comprises a modified sugar moiety, modified nucleobase, and/or modified phosphate group.

91. The method of Claim 90, wherein the modified nucleotide triphosphate comprises a 3'-reversible blocking group (RGB).