IMMOBILIZED TERMINAL NUCLEOTIDYL TRANSFERASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/634,877, filed April 16, 2024; U.S. Provisional Application No. 63/646,600, filed May 13, 2024, and U.S. Provisional Application No. 63/718,950, filed November 11, 2024, the contents of each of which is incorporated by reference herein in its entirety

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The official copy of the Sequence Listing is submitted concurrently with the specification as an XML file, with a file name of "CX10-276WO1 ST26.xml", a creation date of April 15, 2025, and a size of 18,764 bytes. The Sequence Listing filed is part of the specification and is incorporated in its entirety by reference herein.

TECHNICAL FIELD

[0003] The present invention provides immobilized engineered terminal nucleotidyl transferase (TnT) polypeptides useful in template-independent polynucleotide synthesis, as well as compositions and methods of utilizing these immobilized polypeptides.

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 phoshorothioate 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 present 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, engineered TnT enzymes that are able to accept and incorporate NTPs with an extensive range of modifications to highly modified polynucleotide oligo acceptor substrates and that are immobilized to resins or other solid supports and retain polynucleotide synthesis activity under industrial process conditions could help enable an economically feasible synthesis processes.

SUMMARY

[0024] The present invention provides engineered terminal nucleotidyl transferase (TnT) polypeptides immobilized on a solid support that are useful in template-independent polynucleotide synthesis, as well as compositions and methods of utilizing these immobilized polypeptides. The engineered TnTs that are immobilized in the present invention can include 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*. This summary is intended to introduce the subject matter of the present disclosure, but does not cover each and every embodiment, combination, or variation that is contemplated and described within the present disclosure. Further embodiments are contemplated and described by the disclosure of the detailed description, drawings, and claims.

[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, 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 at least one embodiments, the present invention provides a method for preparing an immobilized terminal nucleotidyl transferase (TnT) comprising: (a) preparing a solution comprising an epoxide functionalized solid support and an engineered TnT polypeptide; (b) allowing the solution to incubate at a temperature of about 20 C to about 60 C for about 1 h to about 6 h; and (c) adding an

epoxide quenching reagent to the solution of step (b). In at least one embodiment of this method, a second epoxide quenching reagent is used.

[0028] In at least one embodiment of the method of preparing an immobilized TnT, the solution of step (a) is a buffered aqueous solution at a pH of about 6.5 to about 8.5 containing the engineered TnT polypeptide at a concentration of about 50 mM to about 250 mM; optionally, wherein the buffer in the aqueous solution is selected from 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 buffer concentration is from about 100 mM to about 1000 mM.

[0029] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide quenching reagent is selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and a combination thereof. In at least one embodiment of the method of preparing an immobilized TnT, the second epoxide quenching reagent is selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol,TEoA-HCl, and a combination thereof. In at least one embodiment, the concentration of the epoxide quenching reagent in the solution is about 10 mM to about 1000 mM or from about 20 mM to about 2000 mM.

[0030] In at least one embodiment of the method of preparing an immobilized TnT, the solution of step (c) is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0031] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0032] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0033] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

[0034] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support is a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion),

EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0035] In at least one embodiment of the method of preparing an immobilized TnT, the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) reagent that comprises a 3'-phosphate removable blocking group.

[0036] In at least one embodiment of the method of preparing an immobilized TnT, wherein the engineered TnT polypeptide comprises an amino acid sequence having at least 85% identity to a sequence of an engineered TnT polypeptide of SEQ ID NO: 2, 6, or 8 or a TnT disclosed in WO2024081770A2.

[0037] In another aspect, the present invention includes in at least one embodiment, a composition comprising an immobilized terminal nucleotidyl transferase (TnT) prepared by method of the present disclosure. In at least one embodiment the composition is prepared by the method of: (a) preparing a solution comprising an epoxide functionalized solid support and an engineered TnT polypeptide; (b) allowing the solution to incubate at a temperature of about 20 C to about 60 C for about 1 h to about 6 h; and (c) adding an epoxide quenching reagent to the solution of step (b). In at least one embodiment, the method of preparing the composition additionally comprises a second epoxide quenching reagent.

[0038] The present invention also provides an immobilized terminal nucleotidyl transferase (TnT) comprising an engineered TnT polypeptide and an epoxide-functionalized solid support, wherein the engineered TnT polypeptide is attached to the solid support through a covalent linkage comprising a β-hydroxy-amino, β-hydroxy-ether, β-hydroxy-carboxyl, and/or β-hydroxy-thio, and wherein at least 99% of epoxide groups are quenched. In at least one embodiment, the quenched epoxide groups comprise a covalent linkage with a quenching compound, and optionally, a second quenching compound, selected

from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and a combination thereof.

[0039] In at least one embodiment of the immobilized TnT, the engineered TnT polypeptide is fused with a second polypeptide; optionally, wherein the second polypeptide has inorganic pyrophosphatase (IPP) activity. In at least one embodiment further comprising a second polypeptide attached to the solid support through a covalent linkage; optionally, wherein the second polypeptide has inorganic pyrophosphatase (IPP) activity.

[0040] In at least one embodiment of the immobilized TnT, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0041] In at least one embodiment of the immobilized TnT, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0042] In at least one embodiment of the immobilized TnT, the epoxide functionalized solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

[0043] In at least one embodiment of the immobilized TnT, the epoxide functionalized solid support is a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), aCPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (LGC), IB-SLC(500A)-MPTMS-P1000DGE (LGC), IB-SLC(500A)-GPTMS (LGC), IB-SLC(500A)-MPTMS-P500DGE-MTMS (LGC), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (LGC), IB-SLC(500A)-GPTMS-MTMS, IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0044] In at least one embodiment of the immobilized TnT, the immobilized TnT is capable of template-independent synthesis.

[0045] In at least one embodiment of the immobilized TnT, the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) that comprises a 3'-phosphate removable blocking group.

[0046] In at least one embodiment of the immobilized TnT, the engineered TnT polypeptide comprises an amino acid sequence having at least 85% identity to a sequence of an engineered TnT polypeptide of SEQ ID NO: 2, 6, or 8 or a TnT disclosed in WO2024081770A2.

[0047] In another aspect, the present invention also provides a method for synthesizing an oligonucleotide comprising: contacting an immobilized terminal nucleotidyl transferase (TnT) of the present disclosure with a reaction solution comprising an acceptor oligonucleotide, a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG), and an inorganic pyrophosphatase (IPP), whereby the acceptor oligonucleotide is extended adding at least one donor nucleotide base to its 3' end; and isolating the synthesized extended acceptor oligonucleotide from the reaction solution.

[0048] In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide is not immobilized or linked to a solid support.

[0049] In at least one embodiment of the method for synthesizing, the immobilized terminal nucleotidyl transferase (TnT) comprises a packed resin in a column.

[0050] In at least one embodiment of the method for synthesizing, said contacting comprises flowing the reaction solution through the packed resin in the column.

[0051] In at least one embodiment of the method for synthesizing, the reaction solution comprises about 100 mM TEA buffer, about 1 mM $CoCl_2$, and about 1 μM IPP, and is at about pH 7.8.

[0052] In at least one embodiment of the method for synthesizing, the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG). In at least one embodiment, the 3'-O-RBG is selected from 3'-O-PO_3, 3'-O-NH_2, and 3'-O-NO_2.

[0053] In at least one embodiment of the method for synthesizing, the donor nucleotide triphosphate (NTP) reagent comprises an α-thiophosphate group.

[0054] In at least one embodiment of the method for synthesizing, the donor nucleotide triphosphate (NTP) reagent comprises a 2' modification; optionally wherein the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

[0055] In at least one embodiment of the method for synthesizing, the donor nucleotide triphosphate (NTP) reagent comprises a locked nucleic acid group.

[0056] In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide has a length of at least 3 bp, at least 4 bp, at least 5 bp, at least 6 bp, and at least 7 bp. In at least one embodiment, the acceptor oligonucleotide has a length of between about 3 bp and 10 bp.

[0057] In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one ribonucleotide base.

[0058] In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one phosphorothioate linkage.

[0059] In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one locked nucleic acid (LNA) linkage.

DESCRIPTION OF THE INVENTION

[0060] **FIGS. 1-5** shows results of screening of different resins, substrate conversion in a TnT reaction, flow characteristics, and leaching of TnT enzyme.

[0061] **FIG. 6** illustrates COOH activation (EDC/NHS) chemistry for immobilizing TnT polypeptides.

[0062] **FIG. 7** and **FIG. 8** show results of TnT immobilization on COOH (EDS/NHS) activated resins.

[0063] **FIGS. 9-12** show HPLC chromatograms indicating products formed in different reactions with or without epoxide quenching by varying levels of ethanolamine, L-lysine, and L-cysteine (see Example 3).

[0064] **FIG. 13A** shows percent of oxidation products generated from use of different quenching reagents at 45 °C, and **FIG. 13B** shows percent of oxidation products generated from use of different quenching reagents at RT.

[0065] **FIG. 14** shows characteristics of several different resins with different reactive groups.

[0066] **FIG. 15** shows activity characteristics in a oligonucleotide synthesis reaction of an engineered TnT enzyme immobilized on IBCOV7 resin compared to activity of free engineered TnT enzyme at different reaction conditions.

DESCRIPTION OF THE INVENTION

[0067] 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.

[0068] 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.

[0069] 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.

[0070] 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.

[0071] 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

[0072] 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

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

[0073] 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 αcarbon, "D-Ala" and "L-Ala" designate D-alanine and L-alanine, respectively.

[0074] 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.

[0075] 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

[0076] 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.

[0077] "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.

[0078] "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0079] "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0080] "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.

[0081] "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.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] "Coding sequence" refers to that portion of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0089] "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.

[0090] 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.

[0091] "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., 1997, 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.

[0092] "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.

[0093] "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.

[0094] 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.

[0095] "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.

[0096] "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 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.

[0097] 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

[0098] "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.

[0099] "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.

[0100] "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.

[0101] "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.

[0102] "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.

[0103] "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.

[0104] 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).

[0105] "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.

[0106] "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.

[0107] "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.

[0108] "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.

[0109] "Thermo- and solvent stable" refers to a polypeptide that is both thermostable and solvent stable.

[0110] 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., 1990, 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.

[0111] "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.

[0112] "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.

[0113] "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.

[0114] 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., 19998, 14:372-73; Stenico et al., Nucl. Acids Res., 1994, 222437-46; Wright, Gene, 1990, 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, N.Y. (2001); Uberbacher, Meth. Enzymol., 1996, 266:259-281; and Tiwari et al., Comput. Appl. Biosci., 19997, 13:263-270).

[0115] "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.

[0116] "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.

[0117] "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.

[0118] "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.

[0119] "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.

[0120] "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.

[0121] "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.

[0122] "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**.

[0123] "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).

[0124] "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.

[0125] "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.

[0126] "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^{γ}-, -PH-, -S(O)-, -S(O)2-, -S(O) NR^{γ}-, -$S(O)_2NR^{\gamma}$, and the like, including combinations thereof, where each R^{γ} is independently selected from hydrogen, alkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

[0127] "Amino" refers to the group -NH_2. Substituted amino refers to the group –NHR^{η}, $NR^{\eta}R^{\eta}$, and $NR^{\eta}R^{\eta}R^{\eta}$, where each R^{η} 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.

[0128] "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.

[0129] "Aminocarbonyl" refers to -$C(O)NH_2$. Substituted aminocarbonyl refers to –$C(O)NR^{\eta}R^{\eta}$, where the amino group $NR^{\eta}R^{\eta}$ is as defined herein.

[0130] "Oxy" refers to a divalent group -O-, which may have various substituents to form different oxy groups, including ethers and esters.

[0131] "Alkoxy" or "alkyloxy" are used interchangeably herein to refer to the group –OR^{ξ}, wherein R^{ξ} is an alkyl group, including optionally substituted alkyl groups.

[0132] "Carboxy" refers to -COOH.

[0133] "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.

[0134] "Carboxyalkyl" refers to an alkyl in which one or more of the hydrogen atoms are replaced with one or more carboxy groups.

[0135] "Aminocarbonylalkyl" refers to an alkyl substituted with an aminocarbonyl group, as defined herein.

[0136] "Halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

[0137] "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.

[0138] "Hydroxy" refers to -OH.

[0139] "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.

[0140] "Thiol" or "sulfanyl" refers to –SH. Substituted thiol or sulfanyl refers to –S-R^{η}, where R^{η} is an alkyl, aryl, or other suitable substituent.

[0141] "Sulfonyl" refers to –SO_2-. Substituted sulfonyl refers to –SO_2-R^{η}, where R^{η} is an alkyl, aryl, or other suitable substituent.

[0142] "Alkylsulfonyl" refers to –SO_2-R^{ξ}, where R^{ξ} is an alkyl, which can be optionally substituted. Typical alkylsulfonyl groups include, but are not limited to, methylsulfonyl, ethylsulfonyl, n-propylsulfonyl, and the like.

[0143] "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.

[0144] "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).

[0145] "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.

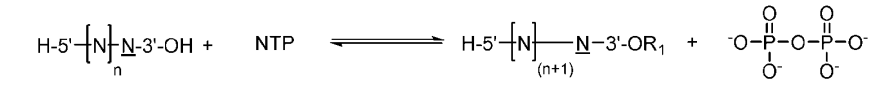
[0146] "Reaction" as used herein refers to a process in which one or more substances or compounds or substrates is converted into one or more different substances, compounds, or processes.

[0147] Template-Independent Synthesis by Engineered TnTs

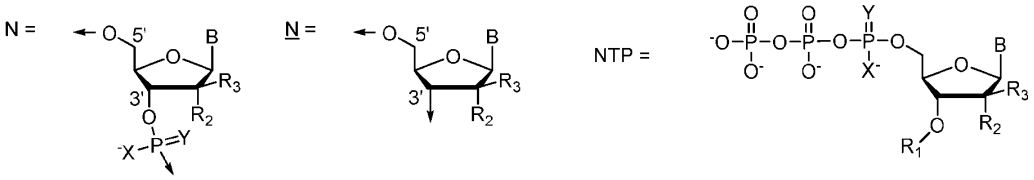
[0148] New methods of efficiently synthesizing high purity strands of DNA, RNA, and other polynucleotides are necessary to overcome the limitations of existing phosphoramidite chemical synthesis methods in order to enable a range of emerging and existing synthetic biology applications.

[0149] 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.

[0150] 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.



where



and

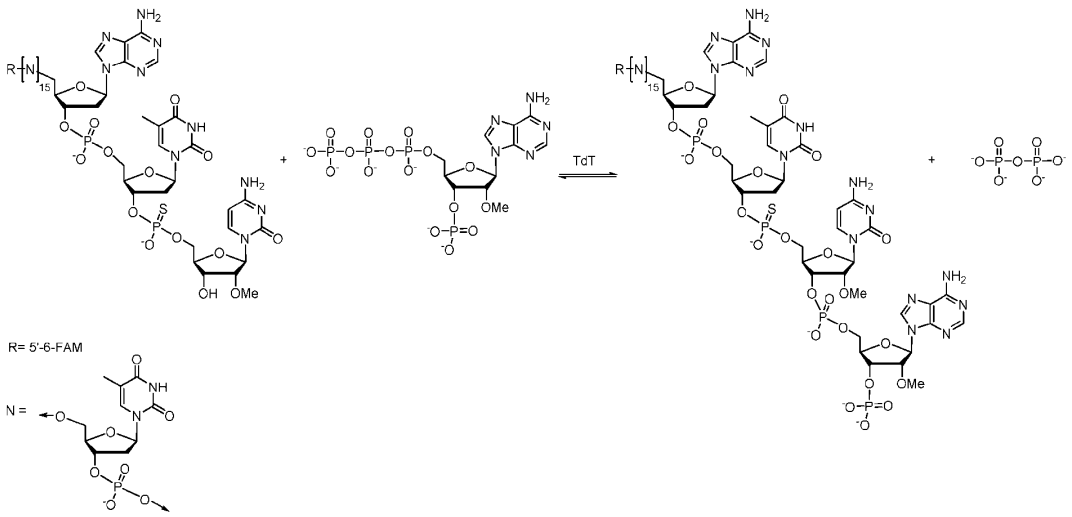
B = adenine, guanine, cytidine, thymine, uracil, pseudouridine, 1-methylpseudouridine

R_1 = benzyl, *O*-nitrolbenzyl, benzoyl, acetyl, cyanoethyl, NO_2, PO_3^{2-}, SO_3^-
R_2 = H, OH, OMe, F, Me, methoxyethyl (MOE)
R_3 = H, OH, OMe, F, Me
X = O,S
Y = O,S

Scheme 1

[0151] 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.

[0152] 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

[0153] 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.

[0154] 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 break down inorganic phosphate and push the reversible TnT reaction toward synthesis.

[0155] 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**.

[0156] 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.

[0157] 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.

[0158] 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

[0159] The present invention is directed to immobilized engineered terminal nucleotidyl transferase (TnT) polypeptides that can be used in template-independent polynucleotide synthesis processes using an NTP-3'-O-RBG or natural or modified NTP substrate, as well as compositions and methods of utilizing these immobilized engineered polypeptides in template-independent oligonucleotide synthesis.

[0160] 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. Other template-independent polymerases (including, but not limited to polyA polymerases, polyU polymerases and terminal uridylytransferases) are also known in the art and may be used to practice the invention. 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. 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, 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.

[0161] The immobilized TnT polypeptides of the present disclosure utilize engineered TnT polypeptides that have residue differences relative to the wild-type polypeptide gene from *Monodelphis domestica* that result in improved properties necessary to develop an efficient TnT enzyme, capable of template-independent synthesis of polynucleotides having a defined sequence. A range of engineered TnT polypeptides have been generated via directed evolution and are described in PCT application WO2024081770A2, published April 18, 2024, which is hereby incorporated by reference herein for all purposes. Further evolution of the engineered TnT polypeptide disclosed as SEQ ID NO: 2 in WO2024081770A2, has been carried out as described in US provisional patent application USSN 63/634,824, filed April 16, 2024, which is hereby incorporated by reference herein for all purposes. The engineered TnT polypeptides disclosed in WO2024081770A2 and USSN 63/634,824 are can be used in the methods of preparing and using immobilized engineered TnT polypeptides of the present disclosure. In at least one embodiment, as described in the Examples and elsewhere herein, the engineered TnT polypeptide of SEQ ID NO: 2, 6, or 8 can be used in the immobilized TnT methods and compositions. It is contemplated that further engineering of these previously described engineered TnT polypeptides can

be used to provide additional TnT polypeptides that can be used in the immobilization methods and compositions of the present disclosure.

[0162] 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. Accordingly, it is contemplated that residue differences resulting in improved immobilized TnT enzymes can be prepared.

[0163] The engineered TnT polypeptides useful in the immobilization methods 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 shown in the Examples. Suitable reaction conditions under which the above-described improved properties of the engineered TnT 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 TnT polypeptide immobilized on a solid support, as further described below and in the Examples.

[0164] Further engineering of TnT polypeptides with improved properties useful in the immobilization methods disclosed herein can be obtained by subjecting the polynucleotide encoding the 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). 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., Nature, 1998, 391:288-291; Crameri, et al., Nat. Biotechnol., 1997, 15:436-438]; Zhang et al., Proc. Nat. Acad. Sci. U.S.A., 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).

[0165] Typically, the engineered 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).

[0166] The clones obtained following mutagenesis and directed evolution can be screened for engineered TnT activity having one or more desired improved enzyme properties (e.g., improved activity when immobilized). 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.

[0167] In some embodiments, the engineered TnT polypeptides useful in the immobilized methods and compositions of the present invention are fusion polypeptides in which the engineered TnT polypeptide is 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 TnT polypeptides described herein can be used with or without fusions to other polypeptides. For example, in one

embodiment of the engineered TnT polypeptides of the present invention, the polypeptide can further comprise an N-terminal truncation of 1-156 amino acids of the polypeptide sequence.

[0168] In some embodiments, the engineered TnT polypeptides useful in the immobilized methods and compositions of the present disclosure can be fused to 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 immobilized for use in the methods could be used as a fusion with a second polypeptide, such as IPP.

Immobilization of Engineered TnT Polypeptides

[0169] The present disclosure is directed to the preparation and use of immobilized engineered TnT polypeptides. Generally, the immobilization process requires contacting the engineered TnT polypeptide in solution with a solid support under conditions suitable for the formation of a covalent or non-covalent linkage between the polypeptide and a functional group on the solid support. The resulting attachment of TnT polypeptide to the support should allow the enzyme to retain its desired activity with a desired substrate. The immobilized TnT polypeptide can then be used in an enzymatic reaction, such as the template independent synthesis of an oligonucleotide, while it is retained on a support that allows facile separation of the desired oligonucleotide reaction products. In at least one embodiment, the immobilized TnT polypeptide is packed in a column allowing a flow-through template-independent oligonucleotide synthesis, wherein the oligonucleotide and NTP substrates are passed through the column, with the desired products collected at the column output. Such flow-through synthesis methods are disclosed in the Examples and elsewhere herein.

[0170] In some embodiments, the immobilized TnT polypeptide is used in a batch reaction, where the immobilized TnT polypeptide on the support is mixed in the reaction solution in a vessel or container, such as with an impeller, with the TnT substrates until the desired level of synthesis is achieved. The immobilized TnT polypeptide can then be separated out from the reaction solution, such as by filtration or centrifugation.

[0171] In some embodiments, the TnT polypeptide can be immobilized non-covalently or covalently on various solid supports or support mediums (e.g., resins, membranes, beads, glass, etc.). In some embodiments, support medium, including solid supports, useful for immobilizing the TnT polypeptides include but are not limited to particles, beads, resins, or membranes, among others, of polyacrylic,

methacrylic, polymethacrylic, phenolic, polystyrene, polyacrylamide, polyamino acid or protein (e.g., gelatin), polysaccharide or cellulosic (e.g., agarose, dextran, chitosan, pectin, etc.), controlled pore glass, and silica (e.g., silicon dioxide).

[0172] In some embodiments, the support medium or solid support is in the form of particles, such as beads, having a particle size range of about 1 μm to about 2000 μm, about 10 μm to about 1700 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. In some embodiments, the particles have a particle size of about 1 μm, about 10 μm, about 50 μm, about 100 μm, about 200 μm, about 300 μm, about 400 μm, about 500 μm, about 1000 μm, about 1500 μm, 1600 μm, 1700 μm, 1800 μm, 1900 μm, or 2000 μm. In some embodiments, the size of the particles are selected for solution flow properties, amount and density of functional groups, surface area, diffusion of substrates, and the like.

[0173] In some embodiments, the particles or resins for immobilization of TnT polypeptide comprises an average pore diameter or pore size of about 100 angstroms (Å) to about 2000 angstroms (Å), 150 angstroms (Å) to about 1800 angstroms (Å), 200 angstroms (Å) to about 1600 angstroms (Å), 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). In some embodiments, the particles comprise an average pore diameter or pore size of about 100 angstroms (Å), 150 angstroms (Å), 200 angstroms (Å), 250 angstroms (Å), 300 angstroms (Å), about 600 angstroms (Å), about 1000 angstroms (Å), about 1500 angstroms (Å), 1600 angstroms (Å), 1700 angstroms (Å), 1800 angstroms (Å), 1900 angstroms (Å), 2000 angstroms (Å), or greater.

[0174] A range of methods of enzyme immobilization are known in the art. These methods use a wide range of solid supports (e.g., resins, membranes, beads, glass, etc.) and result in enzymes that are bound either non-covalently or covalently. Immobilization methods disclosed in the art include, 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.

[0175] Solid supports useful for immobilizing the engineered TdT polypeptides 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.

[0176] Exemplary solid supports useful for immobilizing the engineered TdT polypeptides of the present invention include those disclosed for use as described in the Examples, and include but are not limited to the supports listed in **Table 2** below.

Table 2: Exemplary solid supports for immobilization of engineered TnT							
Manufacturer	**Product #**	**Mode**	**Type**	**Functional Group(s)**	**Low End Particle Size (µm)**	**High End Particle Size (µm)**	**Average Pore Diameter (Å)**
ChiralVision	IB-COV-1	Covalent	Polyacrylic	Low epoxide, high butyl	150	600	
ChiralVision	IB-COV-2	Covalent	Polyacrylic	Epoxide, low butyl	150	600	
ChiralVision	IB-COV-6	Covalent	Polyacrylic	Epoxide, hydroxyethyl	300	710	
ChiralVision	IB-COV-7	Covalent	Polyacrylic	Epoxide	300	600	500
ChiralVision	IB-COV-8	Covalent	Polyacrylic	Epoxide	200	710	
ChiralVision	IB-COV-10	Covalent	Cellulose	Epoxide, anionic	80	120	
ChiralVision	IB-ANI-2	Anionic	Polystyrene	Tertiary Amine	300	700	
ChiralVision	IB-ANI-3	Anionic	Polystyrene	Quaternary Amine	400	1000	
ChiralVision	IB-ANI-5	Anionic	Polyacrylic	Primary, Secondary Amine	300	600	
ChiralVision	IB-ANI-7	Anionic	Polyacrylic	Primary, Secondary Amine	200	710	
ChiralVision	IB-ANI-8	Anionic	Polyacrylic	Primary Amine	200	710	
ChiralVision	IB-ANI-10	Anionic	Polyacrylic	Primary, Secondary Amine	150	600	
ChiralVision	IB-ANI-13	Anionic	Cellulose	Primary, Secondary Amine	80	120	
Duolite	A-7 Freebase	IEX	Phenol/Formaldehyde	Amino			
Duolite	A568	IEX	Phenol/Formaldehyde	Tertiary Amine	150	850	400
Resindion	EC-HFA	Covalent	Methacrylic Polymer	Amino-epoxy	200	500	500
Resindion	EC-BU	Hydrophobic Adsorbent	Methacrylic Polymer	Butyl	200	500	500
Resindion	EC-HA	Ionic	Methacrylic Polymer	Hexamethylamino	200	500	500
Resindion	EC-EP/S	Covalent	Methacrylic Polymer	Epoxide	200	500	500
Resindion	EC-EP	Covalent	Methacrylic Polymer	Epoxide	200	500	500

Table 2: Exemplary solid supports for immobilization of engineered TnT

Manufacturer	Product #	Mode	Type	Functional Group(s)	Low End Particle Size (µm)	High End Particle Size (µm)	Average Pore Diameter (Å)
Resindion	HFA 403/S	Covalent	Methacrylic Polymer	Amino-epoxy	200	500	667
Amberlite	XAD7HP	Adsorbent	Crosslinked Aliphatic Polymer	N/A	430	690	550
Amberlite	FPA51	IEX	Polystyrene	Tertiary Amine	490	690	
Purolite (Lifetech)	ECR8804F	Adsorption	Octadecyl Methacrylate	Octadecyl	150	300	400
Purolite (Lifetech)	ECR8405F	Covalent	Amino C6 Methacrylate	NH_2 (long spacer)	150	300	900
Purolite (Lifetech)	ECR1090F	Adsorption	Macroporous Styrene	N/A	150	300	1000
Purolite (Lifetech)	ECR1604	IEX	Styrene	Tertiary Amine	300	1200	
Purolite (Lifetech)	ECR1504	IEX	Styrene	Tertiary Amine	300	1200	
Purolite (Lifetech)	ECR1640	IEX	Styrene	Quaternary Amine	300	1200	
Sunresin	EMC7025	Covalent	Polyacrylic	Epoxide	150	300	403.25
Sunresin	EMC7014	Covalent	Polyacrylic	Epoxide	150	300	525
Sunresin	EMC7032	Covalent	Polyacrylic	Epoxide	150	300	315
Sunresin	EMC7120/M	Amine	Polyacrylic	Amino, C6 Spacer	300	700	320
Sunresin	EMC7225/M	Amine	Polyacrylic	Amino, C2 Spacer	300	700	620
Sunresin	Chelex 7350	Affinity	Polyacrylic	Iminodiacetate	100	250	
Resindion - Relizyme	HA403/M	Amine	Polyacrylic	Amino	200	500	
Resindion - Relizyme	EA403/M	Amine	Polyacrylic	Amino	200	500	
Resindion - Relizyme	EP403/M	Covalent	Polymethacrylate	Epoxide	200	500	
Resindion - Relisorb	SP600	Adsorbent	Polymethacrylate	Sulfonic	100	200	1500
Resindion - Relisorb	EP600	Covalent	Polymethacrylate	Epoxide	100	200	1500
Resindion - Relizyme	HFA403/S	Covalent	Polymethacrylate	Amino - Epoxy	100	300	500
Sunresin	EMC7042S	Covalent	Polyacrylic	Epoxide	100	300	920

[0177] A wide range of alternative solid supports useful for immobilizing enzymes are known in the art and it is contemplated that these also may be adapted for use with the engineered TnT polypeptides. Some of these alternative solid supports 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).

[0178] In some embodiments, the support material comprises a polymethacrylate resin. Exemplary polymethacrylate resins include HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), and ECR8405F (Purolite).

[0179] In some embodiments, the support material comprises a polyacrylic resin. Exemplary polyacrylic resins include IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EMC7042/M (Sunresin), EMC7042/S (Sunresin), EA403/M (Resindion), and HA403/M (Resindion).

[0180] In some embodiments, the support material comprises a cellulose resin. Exemplary cellulose resins include IB-ANI-13 (ChiralVision BV) and IB-COV-10 (ChiralVision BV).

[0181] In some embodiments, the support material comprises a polystyrene, styrene, or macroporous styrene resin. Exemplary polystyrene, styrene, or macroporous styrene resins include IB-ANI-2 (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), and ECR1640 (Purolite).

[0182] In some embodiments, the support material comprises a controlled pore glass (CPG) resin. Exemplary CPGs include CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), and CPG-21 (LGC).

[0183] In some embodiments, the support material comprises silica. Exemplary silica resins include IB-SLC(500A)-MPTMS-P500DGE (LGC), IB-SLC(500A)-MPTMS-P1000DGE (LGC), IB-SLC(500A)-GPTMS (LGC), IB-SLC(500A)-MPTMS-P500DGE-MTMS (LGC), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (LGC), and IB-SLC(500A)-GPTMS-MTMS.

[0184] In some embodiments, the support material comprises an affinity resin. Exemplary affinity resins include IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), and IB-His-2 Co(II) (ChiralVision).

[0185] In some embodiments, the support material comprises another organic or inorganic material. Additional exemplary support materials comprise A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

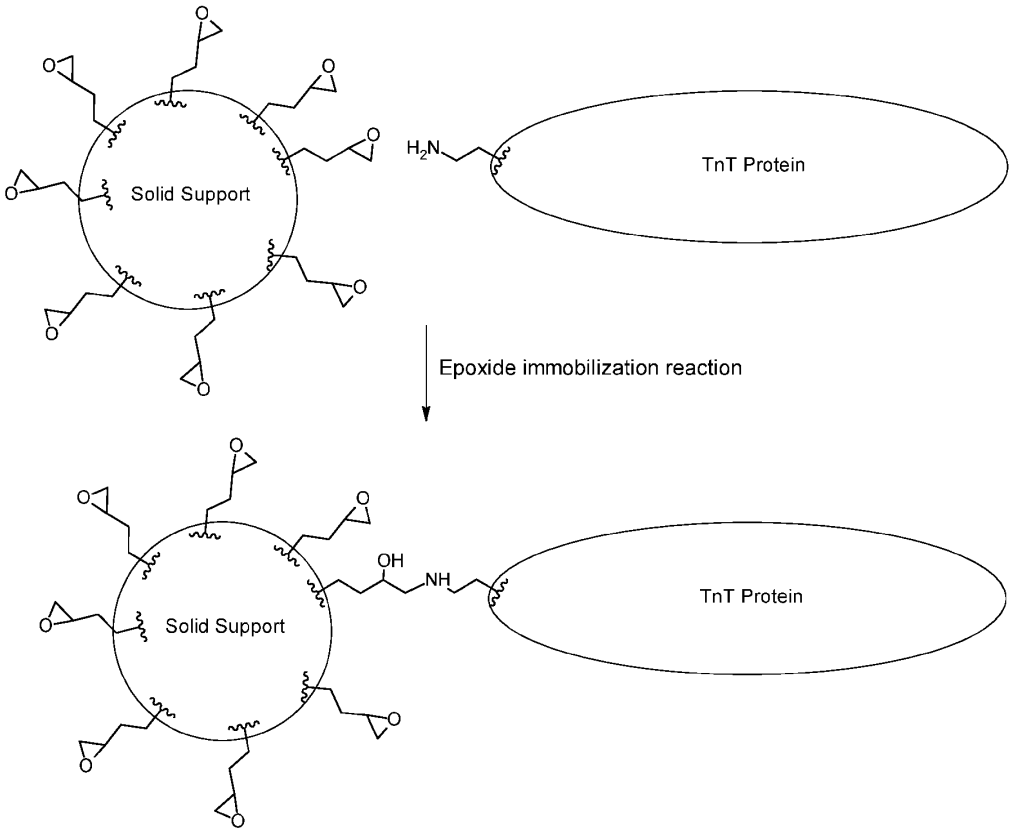
Covalent Attachment

[0186] In some embodiments, the polypeptide is immobilized on the support material by covalent attachment to a reactive chemical group. Exemplary reactive chemical groups include sulfonic, epoxide, epoxy, amino-epoxy, iminodiacetate, amino, primary amine, secondary amine, quaternary amine, tertiary amine, NH2, octadecyl, butyl, high butyl, low butyl, and hydroxyethyl. In some embodiments, the reactive chemical group facilitates the attachment or immobilization of an oligonucleotide.

[0187] In some embodiments, the TnT polypeptide comprises one or more amino acid residues reactive with the reactive chemical group on the support material. In some embodiments, the reactive amino acid is part of the amino acid sequence of the TnT polypeptide, whether the TnT is naturally occurring or an engineered TnT. In some embodiments, reactive amino acids can be added into the TnT amino acid sequence by recombinant methods, such as insertion of cysteine, lysine, aspartic acid, tyrosine, serine, and/or threonine residues. In some embodiments, the TnT polypeptide can be fused to polylysine residues (e.g., 2-12 or more lysine residues), for example, at the amino or carboxy terminus of the TnT.

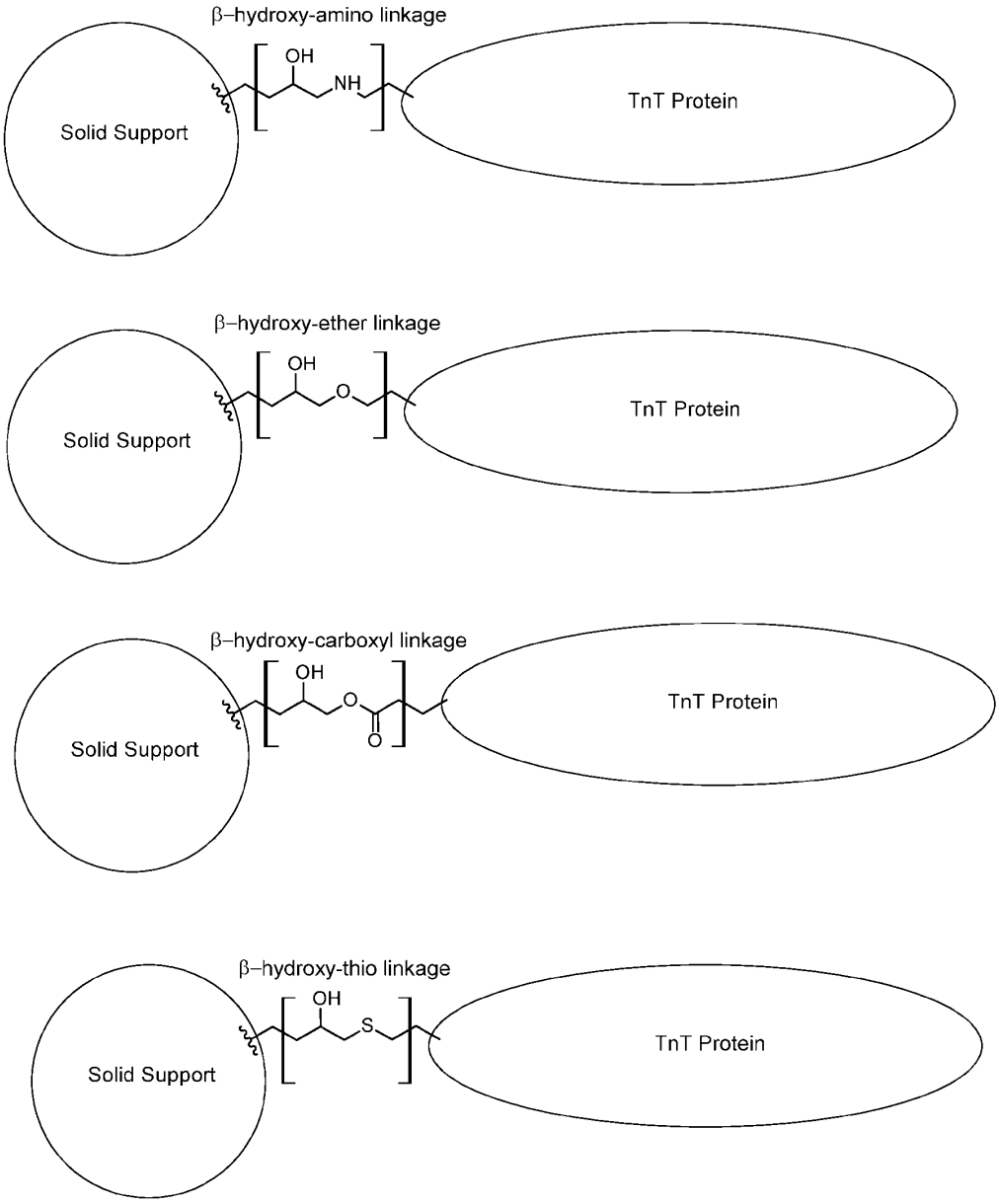
[0188] In some embodiments, the reactive chemical is attached to the resin via a linker. Any suitable linker may be used. Exemplary linkers include ethylamine, alkylene, alkenylene, alkynylene, heteroalkylene, heteroalkenylene, heteroalkynylene, β-hydroxy-amino, β-hydroxy-ether, β-hydroxy-carboxyl, and/or β-hydroxy-thio cyclcoalkyl, heterocycloalkyl, arylene, or heteroarylene based linkers, and the like. In some embodiments the linker comprises a C_2-C_{20}alkylene or polyethylene linker. In some embodiments, the linker is a silane or silanol linker. In some embodiments, the linker is a poly(ethylene glycol) linker, such as (3-mercaptopropyl)trimethoxysilane, poly(ethylene glycol) diglycidyl ether, or (3-glycidyloxypropyl)trimethoxysilane. Various suitable linkers are known in the art.

[0189] In at least one embodiment of the method of preparing an immobilized TnT, the solid support is an epoxide functionalized solid support. In such an embodiment the engineered TnT polypeptide forms a covalent linkage with the solid support. A schematic illustration of immobilization reaction between epoxide-functionalized solid support and a primary amine bearing amino acid residue of an engineered TnT polypeptide to form β-hydroxy amino linkage between solid support and protein as shown in **Scheme 3** below.



Scheme 3

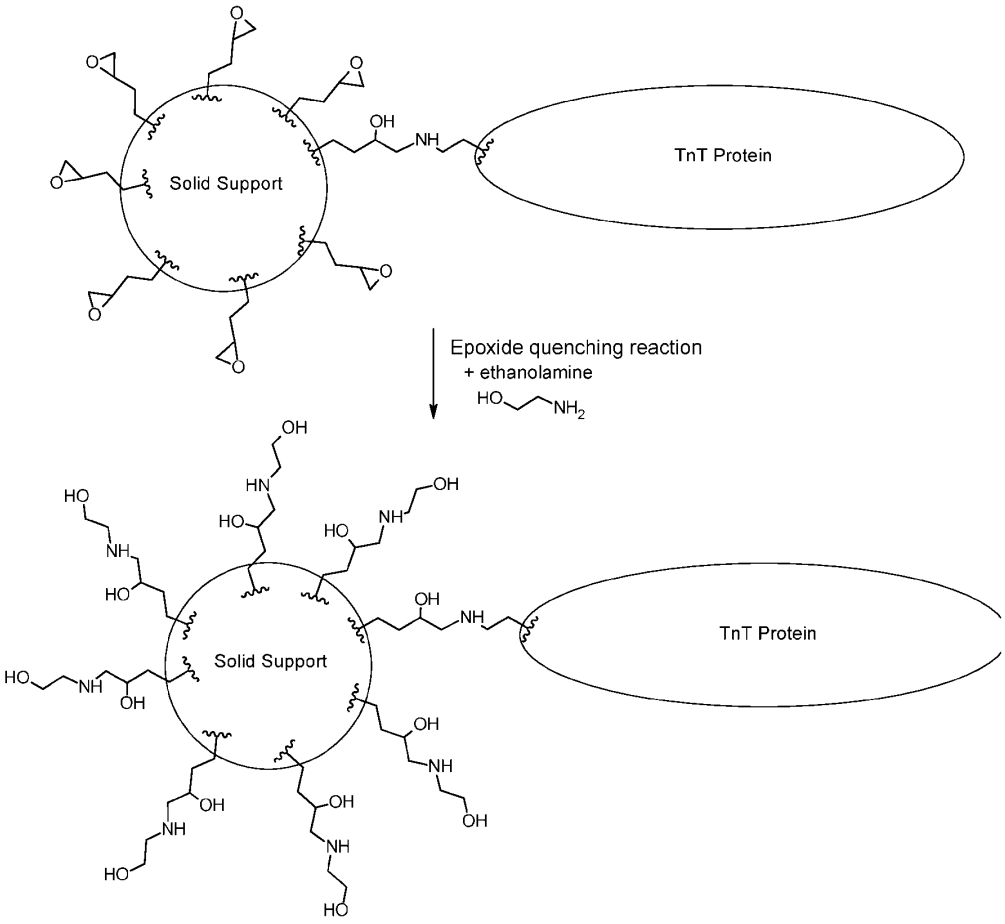
[0190] The four exemplary β-hydroxy linkages that can form in the reaction between an epoxide functionalized solid support and an engineered TnT protein are shown in **Scheme 4** below.



Scheme 4

[0191] Further, as described in the Examples and elsewhere herein, it has been found that an unexpected improvement in the performance of the immobilized TnT polypeptide occurs when immobilization on an epoxide functionalized solid support includes a step of quenching the excess epoxide functional group on the support with one or more quenching reagents. **Scheme 5** shows an exemplary quenching reaction that

use ethanolamine to "quench" the remaining unreacted epoxide groups on a solid support following an epoxide immobilization reaction of TnT protein.



Scheme 5

[0192] Accordingly, in at least one embodiment, the present invention provides a method of preparing an immobilized terminal nucleotidyl transferase (TnT) polypeptide comprising:

contacting an epoxide-functionalized solid support and a TnT polypeptide in solution under suitable conditions for conjugating or immobilizing the TnT polypeptide to the solid support; and

adding an epoxide quenching reagent to the solution to deactivate or cap the epoxide groups on the solid support.

[0193] In at least one embodiment, a method of preparing an immobilized terminal nucleotidyl transferase (TnT) comprises:

(a) preparing a solution comprising an epoxide functionalized solid support and an engineered TnT polypeptide;

(b) allowing the solution to incubate at a temperature of about 20 °C to about 60 °C for about 1 h to about 6 h; and

(c) adding an epoxide quenching reagent to the solution of step (b).

[0194] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, or TEoA-HCl, and any combinations thereof.

[0195] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide quenching reagent is selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl, and any combinations thereof.

[0196] In some embodiments, the concentration of the epoxide quenching reagent used is about 10 mM to about 5000 mM, 500 mM to about 5000 mM, about 10 mM to about 3000 mM, about 100 mM to about 4000 mM, about 500 mM to about 3000 mM.

[0197] In some embodiments, the solution with the added quenching reagent is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0198] In some embodiments, as noted above, the reaction or incubation of the TnT polypeptide and the epoxide functionalized solid support is carried out at 20 °C to 60 °C.

[0199] In some embodiments, the method further comprises removing the quenching reagent by washing the immobilized TnT on the solid support.

[0200] In some embodiments, the wash solution has a pH of about 7, about 7.8, about 8, or about 9.

[0201] In some embodiments, the wash solution is selected from a solution of NaCl/ TEoA-HCL, TEoA-HCL, MOPS, MOPS/NaCl, and NaCl.

[0202] In some embodiments, the wash solution is selected from 500 mM NaCl/50 mM TEoA-HCL, 50 mM TEoA-HCL, 250 mM MOPS, 250 mM MOPS/500 mM NaCl, and 500 mM NaCl.

[0203] In some embodiments, the TnT polypeptide is provided at a concentration of about 0.1 g/L to about 100 g/L; about 1 g/L to about 90 g/L, about 2 g/L to about 80 g/L, 3 g/L to about 70 g/L, about 4 g/L to about 60 g/L, about 5 g/L to about 50 g/L, about 10 g/L to about 40 g/L, or about 20 g/L to about 30 g/L. In some embodiments, the TnT polypeptide is provided at a concentration of about 0.1 g/L, about 1 g/L, about 2 g/L, about 3 g/L, about 5 g/L, about 10 g/L, about 20 g/L, about 30 g/L, about 40 g/L, about 50 g/L, about 60 g/L, about 70 g/L. about 80 g/L, about 90 g/L or about 100 g/L.

[0204] In some embodiments of the method, the solution for reaction of the TnT polypeptide and the epoxide functionalized solid support is a buffered aqueous solution at a pH of about 6.5 to about 8.5. In some embodiments, the buffer in the aqueous solution is selected from 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).

[0205] In some embodiments, the buffer concentration is from about 10 mM to about 2000 mM, 50 mM to about 1500 mM, about 100 mM to about 1000 mM, about 150 mM to about 800 mM, about 200 mM to about 600 mM, or about 300 to about 500 mM. In some embodiments, the buffer concentration is about 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 1000 mM, 1500 mM, or 2000 mM.

[0206] In least one embodiment of this method, the solution for contacting the epoxide functionalized solid substrate and the TnT polypeptide, as in step (a) above, is a buffered aqueous solution at a pH of about 6.5 to about 8.5 containing the engineered TnT polypeptide at a concentration of about 0.1 g/L to about 50 g/L; optionally, wherein the buffer in the aqueous solution is selected from 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 buffer concentration is from about 100 mM to about 1000 mM.

[0207] In some embodiments, a second epoxide quenching reagent is used. In some embodiments, the method further comprises adding a second quenching reagent to the solution of the TnT polypeptide and epoxide functionalized solid support. In some embodiments, the second epoxide quenching reagent is different from the first epoxide quenching reagent. In some embodiments, the second epoxide quenching reagent is used after quenching or capping of the epoxide groups using the first quenching reagent. In some embodiments, the second quenching reagent is added after completion of the treatment with the quenching reagent, i.e., first quenching reagent. In some embodiments, the second quenching reagent is used after removal, e.g., washing, of the first quenching reagent from the immobilized TnT on the solid support.

[0208] In at least one embodiment of the method of preparing an immobilized TnT, the second epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, or TEoA-HCl, or any combinations thereof.

[0209] In at least one embodiment of the method of preparing an immobilized TnT, the second epoxide quenching reagent is selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl, or any combinations thereof.

[0210] In at least one embodiment, the concentration of the second epoxide quenching reagent in the solution is about 10 mM to about 3000 mM, about 50 mM to about 2500 mM, about 100 mM to about 2500 mM, about 200 mM to about 2000 mM, or about 500 mM to about 1000 mM. In some embodiments, the concentration of second quenching reagent in the solution is about 10 mM, about 50 mM, about 100 mM, about 200 mM, about 500 mM, about 1000 mM, about 1500 mM, about 2000 mM, about 2500 mM, or about 3000 mM.

[0211] In some embodiments, the second quenching reagent is reacted in a buffered aqueous solution at a pH of about 6.5 to about 8.5. In some embodiments, the solution comprises a buffer selected from 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).

[0212] In some embodiments, the buffer concentration for use with the second quenching reagent is from about 10 mM to about 2000 mM, 50 mM to about 1500 mM, about 100 mM to about 1000 mM, about 150 mM to about 800 mM, about 200 mM to about 600 mM, or about 300 to about 500 mM. In some embodiments, the buffer concentration is about 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 1000 mM, 1500 mM, or 2000 mM.

[0213] In at least one embodiment, the concentration of the second epoxide quenching reagent in the solution is about 10 mM to about 1000 mM or from about 20 mM to about 2000 mM. In at least one embodiment of the method of preparing an immobilized TnT, the solution of step (c) is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0214] It is to be understood that the treatment of the support medium with quenching reagent following reaction of the TnT polypeptide with the epoxide functionalized support is not limited to two treatments. In some embodiments, the additional treatments of the support medium with the quenching reagent can be carried out multiple times to reduce the levels of epoxide groups on the support medium.

[0215] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises particles, beads, resins, or membranes, among others, comprised of polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, polyacrylamide, polyamino acid or protein (e.g., gelatin), polysaccharide or cellulosic (e.g., agarose, dextran, chitosan, pectin, etc.), controlled pore glass, or silica (e.g., silicon dioxide).

[0216] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises particles comprises polyacrylate, methacrylate, polymethacrylate, amino-epoxy polymethacrylate, phenolate, polystyrene, polysaccharide (e.g., cellulosic), silica, or controlled pore glass.

[0217] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises particles having a particle size range of about 1 μm to about 2000

μm, about 10 μm to about 1700 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. In some embodiments, the epoxide functionalized particles have a particle size of about 1 μm, about 10 μm, about 50 μm, about 100 μm, about 200 μm, about 300 μm, about 400 μm, about 500 μm, about 1000 μm, about 1500 μm, 1600 μm, 1700 μm, 1800 μm, 1900 μm, or 2000 μm. In some embodiments, the size of the particles are selected for solution flow properties, amount and density of functional groups, surface area, diffusion of substrates, and the like.

[0218] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0219] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises particles having an average pore diameter of about 100 angstroms (Å) to about 2000 angstroms (Å), 150 angstroms (Å) to about 1800 angstroms (Å), 200 angstroms (Å) to about 1600 angstroms (Å), 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). In some embodiments, the particles comprise an average pore diameter or pore size of about 100 angstroms (Å), 150 angstroms (Å), 200 angstroms (Å), 250 angstroms (Å), 300 angstroms (Å), about 600 angstroms (Å), about 1000 angstroms (Å), about 1500 angstroms (Å), 1600 angstroms (Å), 1700 angstroms (Å), 1800 angstroms (Å), 1900 angstroms (Å), 2000 angstroms (Å), or greater.

[0220] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0221] In at least one embodiment of the method of preparing an immobilized TnT, the epoxide functionalized solid support is a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC),

aCPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (LGC), IB-SLC(500A)-MPTMS-P1000DGE (LGC), IB-SLC(500A)-GPTMS (LGC), IB-SLC(500A)-MPTMS-P500DGE-MTMS (LGC), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (LGC), IB-SLC(500A)-GPTMS-MTMS, IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0222] The immobilized engineered TnT polypeptides can be prepared using TnT polypeptide 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. In some embodiments, the final immobilized enzyme preparations can be lyophilized, spray-dried, precipitated or be in the form of a crude paste, as further discussed below. In some embodiments, engineered TnT polypeptide is simultaneously purified and immobilized on a solid support.

[0223] In some embodiments of the methods of preparing the immobilized TnT as described herein, the engineered TnT polypeptide may be added to the reaction mixture comprising the functionalized solid support 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).

[0224] In at least one embodiment, a purified or substantially purified engineered TnT polypeptide preparation will be used in the immobilization process and the immobilized enzyme retained as a slurry in an aqueous buffer. Such a resin slurry can then be used to pack a column for use in flow-through oligonucleotide synthesis process.

[0225] In some embodiments, the immobilized engineered TnT polypeptides of the present disclosure 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.

[0226] In some embodiments, the immobilized engineered TnT polypeptides described herein are provided in the form of kits. The immobilized enzymes in the kits may be present individually or as a plurality of immobilized 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.

[0227] In some embodiments, the kits of the present invention include arrays comprising a plurality of different immobilized TnT polypeptides at different addressable position, wherein the different immobilized 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 engineered TnT polypeptides. Such arrays comprising a plurality of engineered polypeptides and methods of their use are known in the art (See e.g., WO2009/008908A2).

Compositions and Immobilized TnT

[0228] In a further aspect, the present disclosure provide a composition of an immobilized TnT disclosed herein. In some embodiments, the present disclosure provides a composition of immobilized TnT polypeptide prepared by any of the methods described herein.

[0229] In some embodiments, the immobilized TnT is combined with other components and compounds to provide compositions and formulations comprising the immobilized TnT as appropriate for different applications and uses.

[0230] In some embodiments, the immobilized on an epoxide functionalized solid support comprising particles, beads, resins, or membranes, among others, comprised of polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, polyacrylamide, polyamino acid or protein (e.g., gelatin), polysaccharide or cellulosic (e.g., agarose, dextran, chitosan, pectin, etc.), controlled pore glass, or silica (e.g., silicon dioxide).

[0231] In some embodiments, the TnT polypeptide is immobilized on a solid support which is an epoxide functionalized solid support or an amino-epoxide functionalized solid support. In some embodiments, the composition comprises an immobilized TnT polypeptide wherein the solid support is a polyacrylate substrate, particularly a hydrophilic polyacrylate support where the TnT polypeptide is covalently attached using epoxide groups on the polyacrylate.

[0232] In some embodiments, the solid support has an average pore diameter of about 250 angstroms (Å) to about 2000 (Å), about 250 angstroms (Å) to about 1500 Å, about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). Other average pore sizes for the solid support include those described herein.

[0233] In some embodiments, the solid support has a particle size range of about 10 μm and 1500 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. Other particles sizes for the solid support include those described herein.

[0234] In some embodiments of the immobilized TnT the covalent linkage is selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

[0235] In some embodiments of the immobilized TnT the covalent linkage comprises at least two types of covalent linkages selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

[0236] In some embodiments, the immobilized TnT comprises quenched epoxide group comprising a covalent linkage with a quenching reagent and, optionally, a second quenching compound, selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0237] In some embodiments, the immobilized TnT comprises quenched epoxide group comprising a covalent linkage with a quenching compound and, optionally, a second quenching compound, selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0238] In some embodiments, TnT is immobilized on a epoxide functionalized solid support comprising a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0239] In some embodiments, the immobilized TnT retains at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 96%, 97%, 98%, 99% or greater activity following attachment to the solid support.

[0240] In some embodiments, the immobilized TnT exhibits less than 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.25% or less of the total wt % bound TnT polypeptide leaching off the solid support after at least 24 hr of storage at 4 °C.

[0241] In some embodiments, the immobilized TnT exhibits less than 5%, 4%, 3%, 2%, 1% or less of the total wt % bound TnT leaching off the solid support after wash at or above pH 7.00

[0242] In some embodiments, the immobilized TnT has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of total wt % bound TnT remaining attached to the solid support after a wash at or above pH 7.5.

Methods of Using Immobilized Engineered TnT Polypeptides

[0243] In some embodiments, the immobilized TnT enzymes described herein find use in processes for conversion of one or more suitable substrates to a product.

[0244] In some embodiments, the immobilized engineered TnT polypeptides disclosed herein can be used in a process for the conversion of the acceptor oligonucleotide substrate and an NTP substrate with a 3'-phosphate removable blocking group (i.e., "NTP-3'-O-RBG" substrate) or a natural or a modified NTP substrate to a product comprising the acceptor oligonucleotide substrate extended by one nucleotide.

[0245] In at least one embodiment, the method for synthesizing an oligonucleotide comprises:

(a) contacting an immobilized terminal nucleotidyl transferase (TnT) of the present disclosure with a reaction solution comprising an acceptor oligonucleotide, a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG), and an inorganic pyrophosphatase (IPP), whereby the acceptor oligonucleotide is extended adding at least one donor nucleotide base to its 3' end; and

(b) isolating the synthesized extended acceptor oligonucleotide from the reaction solution.

[0246] Generally, in the method the acceptor oligonucleotide substrate is in solution and is not immobilized or linked to a solid support as it is in conventional solid phase oligonucleotide synthesis processes. The immobilized terminal nucleotidyl transferase (TnT) polypeptide may be immobilized on solid support that is a resin. This resin can be packed in a column and used in a method wherein the acceptor oligonucleotide substrate and NTP substrate are in solution. Accordingly, in the method for synthesizing, the contacting of the substrates with the enzymes can comprise flowing the reaction solution through a column packed with a resin comprising the immobilized engineered TnT polypeptide.

[0247] In at least one embodiment of the method for synthesizing, the reaction solution can comprise a buffer of about 100 mM TEA or TEoA, about 1 mM $CoCl_2$, at about pH 7.8. As noted elsewhere herein, the presence of the enzyme IPP can further facilitate the TnT catalyzed oligonucleotide extension reaction by cleaving pyrophosphate formed in the solution as a side-product. Accordingly, in some embodiments, the reaction solution can further comprise about 1 μM IPP.

[0248] 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 immobilized 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 immobilized 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.

[0249] 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 an immobilized engineered TnT.

[0250] 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 at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one ribonucleotide base. In some embodiments, the oligo acceptor substrate may comprise one or more additional modifications, such as a phosphorothioate linkage or a locked nucleic acid.

[0251] In some 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. In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide has a length of at least 3 bp, at least 4 bp, at least 5 bp, at least 6 bp, and at least 7 bp. In at least one embodiment, the acceptor oligonucleotide has a length of between about 3 bp and 10 bp.

[0252] 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.

[0253] In at least one embodiment of the method for synthesizing, the immobilized engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG). In at least one embodiment, the 3'-O-RBG is selected from 3'-$O-PO_3$, 3'-$O-NH_2$, and 3'-$O-NO_2$. Accordingly, in at least one embodiment of the method for synthesizing, the donor nucleotide triphosphate (NTP) reagent comprises a 2' modification; optionally

wherein the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

[0254] 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. Accordingly, in at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one locked nucleic acid (LNA) linkage.

[0255] 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.

[0256] 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.

[0257] 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.

[0258] In some embodiments, the oligo acceptor substrate comprises one or more nucleotide sequences selected from the following 5'-6-FAM-T10mCmCmCmUfA, 5'-6-FAM-T11AmC*mA*mG, 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T15mAmUmCmU, 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T36fCmAmUmC, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41*fC*mAfA, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T41mAmUmCmU, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T46mUmCmUmU, 5'-6-FAM-T51fGmUfGmU, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA, 5'-6-FAM-T9mAmAmAmUmCmU, 5'P-mUmCmU, mAfGmUfG, mAmAmAmAmUmCmU, mAmAmAmUmCmU, mAmAmUmCmU, mAmUmCmU, mC*mA*mGmA, mGmUmC*mC, mUmCmU, T8fCmAmUmC, T8mCfCmCfG, T8mCfCmGfG, T8mUfGmUfC, as further described in the Examples and the accompanying sequence listing. These

embodiments are intended to be non-limiting. Any suitable oligo acceptor substrate finds use in the present invention.

[0259] 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.

[0260] 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.

[0261] 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 α phosphate oxygen for an α phosphorothioate group with a sulfur atom which allows 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. Accordingly, in at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one phosphorothioate linkage.

[0262] 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 the immobilized enzyme activity, stability of the immobilized 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.

[0263] 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.

[0264] In some embodiments, the improved activity of the immobilized 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.

[0265] In some embodiments, the immobilized 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 immobilized 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.

[0266] In some embodiments, the immobilized 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.

[0267] In some embodiments, the immobilized 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.

[0268] 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.

[0269] 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.

[0270] 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.

[0271] 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.

[0272] 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.

[0273] 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 immobilized 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 immobilized TnT enzyme under the reaction conditions. Appropriate co-solvent systems can be readily identified by measuring the enzymatic activity of the specified immobilized engineered TnT enzyme with a defined substrate of interest in the candidate solvent system, utilizing an enzyme activity assay, such as those described herein.

[0274] 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).

[0275] 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-sorbitan monostearate, hexadecyl dimethylamine, 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.

[0276] 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.

[0277] The quantities of reactants used in the immobilized 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.

[0278] 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.

[0279] The synthesis 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.

[0280] Any of the processes disclosed herein using the immobilized 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 immobilized 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.

[0281] 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 immobilized 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 immobilized enzyme, reduce product inhibition, shift reaction equilibrium to formation of the desired product.

[0282] In some embodiments, the present disclosure provides an immobilized engineered TnT, wherein said immobilized 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 immobilized 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 NO: 2, 6, or 8 or to an engineered TnT polypeptide disclosed in WO2024081770A2.

[0283] 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.

[0284] Various features and embodiments of the invention are illustrated in the following representative examples, which are intended to be illustrative, and not limiting.

EXAMPLES

[0285] 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.

[0286] 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); cm (centimeters); mm (millimeters); μM and μη (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).

Abbreviations for modified nucleotides

Abbreviations for modified nucleotides	
Alias	**Modification**
5'-FAM	5'-6-carboxyfluorescein modification
T{n}	poly-thymidine of length n
3'P	3'-phosphorylation modification
mA	2'-methoxyadenosine
mC	2'-methoxycytidine
mG	2'-methoxyguanosine
mU	2'-methoxyuridine
fA	2'-fluoroadenosine
fC	2'-fluorocytidine
fG	2'-fluoroguanosine
fU	2'-fluorouridine
rA	adenosine ribonucleotide
rC	cytidine ribonucleotide
rG	guanosine ribonucleotide

Abbreviations for modified nucleotides	
rU	uridine ribonucleotide
*	phosphorothioate linkage
5'-P	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

Modified oligonucleotides referenced in Examples

Modified oligonucleotides referenced in Examples	
Alias	**Sequence**
4A0	dAdAdAdAmC*mA*mG
	mC*mA*mGmAmAmAfCmA-3'P
4A0m	dAdAdAdAfGmUfG

Example 1

Screening of TnT Immobilization Resins

[0287] This example illustrates a study of different resins for the immobilization of engineered TnT polypeptides that can be used for oligonucleotide synthesis.

Materials and Methods

[0288] A range of approximately 40 non-covalent adsorptive and covalent resins were screened for their immobilization of engineered TnT polypeptides. Three main categories of resins were screened: (1) epoxide functionalized resins forming covalent linkages; (2) amine functionalized resins forming covalent linkages (these also required crosslinker pre-treatment with glutaraldehyde and/or glyoxal); and (3) adsorptive non-covalent binding resins. A chart summarizing the various resins screened and their characteristics is shown in **Table 2** (above).

[0289] Screening was carried out in two phases. The initial 40 resins were screened in a higher throughput 96-well assay. Then the best performing 15 resins of the 40 in the initial screen were screened again using a gram-scale quantity.

[0290] A. The general scheme for initial higher throughput screening the resins was as follows:

(1) Load ~20 to 30 mg resin into a 96-well plate.

(2) Immobilize the engineered TnT polypeptide SEQ ID NO: 2 at a concentration of 5 wt% . Crosslinker was also added if pre-treatment of the resin was required.

(3) Add to each well a reaction mixture of the acceptor oligonucleotide substrate dAdAdAdAfGmUfG and the NTP substrate with 3'-O-RBG, mUQP to the well.

(4) Collect reaction products and analyze for expected 3'-extended oligonucleotide product (dAdAdAdAfGmUfGmU-3'P) at least two time points.

B. The general scheme for the secondary gram-scale screening the top performing 15 resins was as follows:

(1) The engineered TnT polypeptide SEQ ID NO: 2 at a target weight of 5 wt% was incubated overnight in 0.5 M TEoAHCl, pH 7.8, at 25 °C with 1 gram of each of the top 15 resins.

(2) Wash with 0.5 M NaCl/ 50 mM TEoA, pH 7.8 buffer at 40 °C. Loading of the various resins with the TnT polypeptide was analyzed as shown in **FIG. 1**. Wash fractions were separately analyzed for the presence of leached TdT polypeptide as described below.

(3) Flow the acceptor oligonucleotide, dAdAdAdAmC*mA*mG and the NTP substrate, mAQP over the resin at ~10 CV/h, 0.08 mL/min. Substrate conversion rate to dAdAdAdAmC*mA*mG*mA-3'P was determined an equilibrated conversion average of ~3 fractions. No sign of drop in substrate conversion was observed after ~20 CV.

(4) Measure product solution to determine amount of oligonucleotide retained on resin after flow.

C. The general scheme for measuring TnT polypeptide leaching from resins was as follows:

(1) 50 mM TEoAHCl, pH 7.8, at 40 °C was flowed over the column packed with 300 mg TnT-loaded resin (prepared as described above).

(2) Two column-volumes (CV) (1 CV is ~430 μL) of fractions were collected for a total of ~24 CV.

(3) Collected fractions were analyzed for residual TnT activity using the CE assay and calibration curve. CE assay conditions: 10 μM of 5'-FAM-TTTTTTTTTTTTTTTmCmAmC ; 20 μM of mAQP NTP substrate; 0.25 μM iPP (SEQ ID NO: 4); Buffer conditions: 50 mM TEoA, 0.25 mM $CoCl_2$, pH 7.8, at 50 °C; reaction time of 0.5 h.

D. The general scheme for measuring oligonucleotide retention by resins was as follows:

[0291] Each resin was loaded at ~5 wt% with the engineered TnT polypeptide then incubated with the oligonucleotide substrate, 0.4 mM dAdAdAdAmC*mA*mG under the flow reaction buffer: 50 mM TEoAHCl, pH 7.8, at 40 °C.

Results

[0292] **FIGS. 1-5** provide plots and charts summarizing the results of the resin screening studies, substrate conversion rates in flow reactions, and leaching studies.

[0293] As shown by the oligonucleotide retention study results summarized in **FIG. 6**, amine based resins showed much higher oligonucleotide retention. The SunResin EMC70xx series of resins showed high rates of oligonucleotide retention, perhaps due to larger surface area, than IBCOV7. Attempts to quantitate washes of packed resins after oligonucleotide incubation with HPLC were difficult to interpret and suggest that on-resin chemistry, perhaps related to PS linkage oxidation, occurs with the dAdAdAdAmC*mA*mG oligonucleotide. In general, the amine resins did not release much oligonucleotide product with just a buffer wash except for resin, EA403. For example, A568 did not release the oligonucleotide.

Conclusions

[0294] The gram-scale immobilization of engineered TnT polypeptides was carried out on a wide range of solid support resins. It was found that the TnT polypeptides will leach to some extent when immobilized on an affinity resin solid support. Leaching was not a significant problem when the TnT is covalently attached. Accordingly, covalent attachment to a solid support is preferred for immobilizing the engineered TnT to minimize loss of enzyme when used in an oligonucleotide synthesis process.

Example 2

TnT Immobilization Using COOH Activation (EDC/NHS) Chemistry

[0295] This example illustrates a study of the use of covalent "COOH activation" (or "EDC/NHS") chemistry or immobilizing engineered TnT polypeptides. **FIG. 6** provides a schematic illustration of this type of covalent attachment chemistry for immobilizing a TnT. A series of EDC/NHS resins for ability to retain immobilized TnT with activity in carrying out an oligonucleotide synthesis reaction.

Materials and Methods

[0296] Incubated affinity resins, matrixed against a 2x dilution series of EDC/NHS solution in 50 mM MES pH 6.1 (4h) to explore super-stoichiometric and sub-stoichiometric activation. Washed resins with MES pH 6.1, then incubated with 5 wt% TnT of SEQ ID NO: 2 O/N in MOPS pH 7.0. Washed resins 3x with 0.5 M NaCl / 50 mM TEoAHCl (pH 7.8), then 3x with 50 mM TEoAHCl (pH 7.8). This resin was tested for activity in the conversion of dAdAdAdAfGmUfG and mUQP to dAdAdAdAfGmUfGmU-3'P.

Results

[0297] Results of the EDC/NHS resin studies are shown in **FIG. 7** and **FIG. 8**. The best immobilization conditions for COOH activation used the highest NHS/EDC concentration of 32 mmole EDC per gram resin, and ~1000x more coupling reagent than COOH. Hydrolysis of the NHS-ester is a problem at pH 7.0 (1/2 life of ~4h), but going to a lower pH was not possible because TnT was not soluble at pH 6.0. A small amount of TnT protein precipitation was observed even at pH 7.0. EA403 showed the highest level of oligonucleotide retention. All other resins had similar retention. It was also observed that old IB-COV-7 resin (sitting at 4 °C for ~1 month) exhibited about ½ the activity at 2h vs fresh. In contrast, the old IB-HIS-2-Co(II) affinity immobilized enzyme (sitting at 4 °C for ~1 month) exhibited the same activity as freshly immobilized.

Example 3

TnT Immobilization Using Epoxide Chemistry with Quenching

[0298] This example illustrates a study of immobilizing engineered TnT polypeptides using the epoxide functionalized resin, IB-COV7, followed by epoxide quenching to reduce unwanted oxidation.

Materials and Methods

[0299] Engineered TnT polypeptide of SEQ ID NO: 6 (500 mM TEoA-HCl, pH 7.8 at 4 mg/mL) was immobilized on 4 g of the epoxide functionalized resin, IB-COV-7, at a target loading of 4 wt%. The A280 of the TnT loading solution measured before and after immobilization showed ~3.2 wt% loading of TnT.

[0300] The IB-COV-7 with immobilized TnT was treated for 5 h at room temperature and 45 °C with the quenching compounds ethanolamine, L-lysine and L-cysteine to try to quench/cap the remaining epoxide groups. The concentrations of quenching compounds tested were: 0.01 M, 0.1 M, or 1 M ethanolamine; 1 mM, 10 mM, or 100 mM L-Lysine; 2 mM, 20 mM, 200 mM L-Cysteine. used in shown in **Table 3** below.

Table 3

Ethanolamine	L-Lysine	L-Cysteine
1 M	100 mM	200 mM
0.1 M	10 mM	20 mM
0.01 M	1 mM	2 mM

[0301] An oligonucleotide extension reaction using the immobilized TnT was run, converting dAdAdAdAmC*mA*mG and mAQP to dAdAdAdAmC*mA*mG at 50 °C with timepoints taken at t = 1, 3, and 22 h.

[0302] As a control, the IB-COV-7 with immobilized TnT was also treated with just buffer for 5 h at room temperature and 45 °C.

[0303] A control extension reaction using free TnT was also run at an equivalent enzyme amount for 18 h.

[0304] Resins were either used directly after completion of immobilization procedure (unquenched) or were subjected to a quench step by incubating in solution containing glycine (3M) and NaCl (5M) at room temperature for 18 h, at a volume of 10 mL quench solution per 1.0 g of resin. Afterward, the resin was washed three times with a solution of MOPS (250 mM, pH 8), at room temperature and a volume of 10 mL wash solution per 1.0 g of resin.

[0305] The resin was then used to catalyze the addition of mAQP to mC*mA*mGmAmAmAfC according by combining with 10 mL reaction buffer per mg of resin, under the following conditions: NQP (1.5 mM), CoCl2 (1.0 mM), oligonucleotide acceptor (1.0 mM), MOPS (250 mM, pH 8.0.). The reaction product after 0.5 h was analyzed by HPLC.

[0306] A 2 to 10 µL aliquot is removed from the reaction and diluted to a concentration of 25-50 µM oligonucleotide using 1 mM EDTA (pH 8) in RO water as diluent. A 10 µL portion from the diluted samples is then injected onto an Ultimate 3000 HPLC system using a PAL autosampler according to the method outlined in **Table 3.3**,

Table 3.3. Oligonucleotide Analysis on Oligonucleotide C18 Column

Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Waters ACQUITY Premier Oligonucleotide C18 Column, 2.1x100 mm, 1.7 µm
Mobile Phases	A: 100 mM hexafluoroisopropanol, 22.5 mM dibutylamine, 1% methanol in water B: 100 mM hexafluoroisopropanol , 22.5 mM dibutylamine, 95% methanol in water
LC Gradient	- 0 – 1.0 min: 5.0% B - 1.0 – 2.0 min: 5.0 – 45.0% B - 2.0 – 10.0 min: 45.0-65.0% B - 10.0-10.1 min: 65.0-5.0% B - 10.1-14.0 min: 5% B
Flow rate	0.25 mL/min
Run time	14.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Table 4 Activity of TdT of SEQ ID NO: 8 Immobilized on Epoxide Functionalized Resins		
Resin	**Quenched or Unquenched**	**Conversion to Singly Extended Product**
CPG-19	Unquenched	++++
CPG-20	Unquenched	++++
CPG-21	Unquenched	+
Silica A	Unquenched	++++
Silica B	Unquenched	++++
Silica C	Unquenched	+
Silica D	Unquenched	++++
Silica E	Unquenched	++++
Silica F	Unquenched	+++
IB-COV-7	Unquenched	+++
CPG-19	Quenched	++++
CPG-20	Quenched	++++
CPG-21	Quenched	+
Silica A	Quenched	+
Silica B	Quenched	+
Silica C	Quenched	+
Silica D	Quenched	+
Silica E	Quenched	+
Silica F	Quenched	+
IB-COV-7	Quenched	++
Percent conversion to singly extended product calculated as the AUC (area under curve) of the HPLC chromatogram peak corresponding to mC*mA*mGmAmAmAfCmA-3'P divided by the total AUC of all oligo peaks in the chromatogram, and defined as follows: "+" 40 to 70%, "++" 70 to 90%, "+++" 90 to 95%, and "++++" 95 to 100%.		

Results

[0307] **FIGS. 9, 10, 11,** and **12** show HPLC profiles indicating products formed in different reactions with or without epoxide quenching by varying levels of ethanolamine, L-lysine, and L-cysteine.

[0308] As shown by the results depicted in **FIG. 9**, 0.01 and 0.1 M ethanolamine have greater peak areas for both the oxidation products and dAdAdAdAmC*mA*mGmA-3'P than no quenching. But 1 M ethanolamine shows minimal product peaks (even though dAdAdAdAmC*mA*mG has disappeared).

[0309] As shown by the results depicted in **FIG. 10**, 10 and 100 mM L-lysine have greater peak areas for both the oxidation products and dAdAdAdAmC*mA*mGmA-3'P. No differences were observed between no quench and any of the L-lysine concentrations.

[0310] As shown by the results depicted in **FIG. 11**, 20 and 200 mM L-cysteine have greater peak areas for dAdAdAdAmC*mA*mGmA-3'P and smaller peak areas for the oxidation products than no quenching. At 100 mM the oxidation peaks are roughly equal to the free TnT.

[0311] As shown by the results depicted in **FIG. 12**, at 200 mM L-cysteine, the temperature of the quenching treatment does not appear to have a significant effect. At 20 mM L-cysteine, the 45 °C quenching treatment appears to prevent the formation of the oxidation product more than room temperature quenching.

[0312] The percentage of the products that were oxidized was calculated according to the following formula:

$$\% = \frac{Oxdized\ products\ peak\ area}{Ox + 4A1P\ peak\ areas}$$

[0313] **FIG. 13A** and **FIG. 13B** show plots depicting the calculated amounts of oxidized products produced with 45 °C or room temperature quenching. 200 mM L-cysteine at RT and 45 °C and 20 mM L-cysteine show similar calculated percentages of the oxidized products as the free TnT.

Conclusions

[0314] Quenching the resin, IP-COV-7 after TnT immobilization, with ~200 mM L-cysteine shows promise for preventing oxidation of the phosphorothioate linkages present in the oligonucleotide.

[0315] 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.

[0316] 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. A method for preparing an immobilized terminal nucleotidyl transferase (TnT) comprising:

(a) preparing a solution comprising an epoxide functionalized solid support and an engineered TnT polypeptide;

(b) allowing the solution to incubate at a temperature of about 20 °C to about 60 °C for about 1 h to about 6 h;

(c) adding an epoxide quenching reagent to the solution of step (b); and optionally,

(d) adding a second epoxide quenching reagent to the solution comprising an epoxide functionalized solid support and an engineered TnT polypeptide.

2. The method of claim 1, wherein the solution of step (a) is a buffered aqueous solution at a pH of about 6.5 to about 8.5 containing the engineered TnT polypeptide at a concentration of about 0.1 g/L to about 50 g/L.

3. The method of claim 2, wherein the buffer in the aqueous solution is selected from 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 buffer concentration is from about 100 mM to about 1000 mM; optionally, wherein the buffer comprises 100 mM TEoA at pH 7.8.

4. The method of any one of claims 1-3, wherein the epoxide quenching reagent and, optionally, the second epoxide quenching, is selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and a combination thereof.

5. The method of any one of claims 1-4, wherein the concentration of the epoxide quenching reagent or the second epoxide quenching (if present) in the solution is about 10 mM to about 1000 mM.

6. The method of any one of claims 1-5, wherein the solution of step (c) is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

7. The method of any one of claims 1-6, wherein the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

8. The method of any one of claims 1-7, wherein the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

9. The method of any one of claims 1-8, wherein the epoxide functionalized solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

10. The method of any one of claims 1-9, wherein the epoxide functionalized solid support is a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

11. The method of any one of claims 1-10, wherein the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) reagent that comprises a 3'-phosphate removable blocking group.

12. The method of any one of claims 1-11, wherein the engineered TnT polypeptide comprises an amino acid sequence having at least 85% identity to a sequence of an engineered TnT polypeptide of SEQ ID NO: 2, 6, or 8 or a TnT disclosed in WO2024081770A2.

13. An immobilized terminal nucleotidyl transferase (TnT) prepared by the method of any one of claims 1-12.

14. An immobilized terminal nucleotidyl transferase (TnT) comprising an engineered TnT polypeptide and an epoxide-functionalized solid support, wherein the engineered TnT polypeptide is

attached to the solid support through a covalent linkage comprising a β-hydroxy-amino, β-hydroxy-ether, β-hydroxy-carboxyl, and/or β-hydroxy-thio, and wherein at least 99% of epoxide groups are quenched.

15. The immobilized TnT of claim 14, wherein the quenched epoxide groups comprise a covalent linkage with a quenching compound and, optionally, a second quenching compound, selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and a combination thereof.

16. The immobilized TnT any one of claims 14-15, wherein the engineered TnT polypeptide is fused with a second polypeptide; optionally, wherein the second polypeptide has inorganic pyrophosphatase (IPP) activity.

17. The immobilized TnT of any one of claims 14-16, further comprising a second polypeptide attached to the solid support through a covalent linkage; optionally, wherein the second polypeptide has inorganic pyrophosphatase (IPP) activity.

18. The immobilized TnT of any one of claims 14-17, wherein the immobilized TnT is capable of template-independent synthesis.

19. The immobilized TnT of any one of claims 14-18, wherein the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

20. The immobilized TnT of any one of claims 14-19, wherein the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

21. The immobilized TnT of any one of claims 14-20, wherein the epoxide functionalized solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

22. The immobilized TnT of any one of claims 14-21, wherein the epoxide functionalized solid support is a resin selected from HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), EMC7042/M (Sunresin), EMC7042/S (Sunresin), IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16

(LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

23. The immobilized TnT of any one of claims 14-22, wherein the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) that comprises a 3'-phosphate removable blocking group.

24. The immobilized TnT of any one of claims 14-23, wherein the engineered TnT polypeptide comprises an amino acid sequence having at 85% identity to a sequence of an engineered TnT polypeptide of SEQ ID NO: 2, 6, or 8 or a TnT disclosed in WO2024081770A2.

25. A method for synthesizing an oligonucleotide comprising:

contacting an immobilized terminal nucleotidyl transferase (TnT) of any one of claims 13-24 with a reaction solution comprising an acceptor oligonucleotide, a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG), and an inorganic pyrophosphatase (IPP), whereby the acceptor oligonucleotide is extended adding at least one donor nucleotide base to its 3' end; and

isolating the synthesized extended acceptor oligonucleotide from the reaction solution.

26. The method of claim 25, wherein the acceptor oligonucleotide is not immobilized or linked to a solid support.

27. The method of any one of claims 25-26, wherein the immobilized terminal nucleotidyl transferase (TnT) comprises a packed resin in a column.

28. The method of claim 27, wherein said contacting comprises flowing the reaction solution through the packed resin in the column.

29. The method of any one of claims 25- 28, wherein the reaction solution comprises about 100 mM TEA buffer, about 1 mM $CoCl_2$, and about 0.05 μM to 10 μM IPP, and is at about pH 7.8.

30. The method of any one of claims 25-29, wherein the engineered TnT polypeptide has an activity capable of catalyzing phosphodiester or phosphorothioate linkage formation between a 3'-hydroxyl group of an acceptor oligonucleotide and a donor nucleotide triphosphate (NTP) with a 3' removable blocking group (3'-O-RBG).

31. The method of any one of claims 25-30, wherein the 3'-O-RBG is selected from 3'-O-PO_3, 3'-O-NH_2, and 3'-O-NO_2.

32. The method of any one of claims 25-31, wherein the donor nucleotide triphosphate (NTP) reagent comprises an α-thiophosphate group.

33. The method of any one of claims 25- 32, wherein the donor nucleotide triphosphate (NTP) reagent comprises a 2' modification; optionally wherein the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

34. The method of any one of claims 25-33, wherein the donor nucleotide triphosphate (NTP) reagent comprises a locked nucleic acid group.

35. The method of any one of claims 25-34, wherein the acceptor oligonucleotide has a length of at least 3 bp, at least 4 bp, at least 5 bp, at least 6 bp, and at least 7 bp.

36. The method of any one of claims 25-35, wherein the acceptor oligonucleotide has a length of between about 3 bp and 10 bp.

37. The method of any one of claims 25-36, wherein the acceptor oligonucleotide comprises at least one ribonucleotide base.

38. The method of any one of claims 25-37, wherein the acceptor oligonucleotide comprises at least one phosphorothioate linkage.

39. The method of any one of claims 25-38, wherein the acceptor oligonucleotide comprises at least one locked nucleic acid (LNA) linkage.