SYSTEMS AND METHODS FOR ENZYMATIC OLIGONUCLEOTIDE SYNTHESIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/633,644, filed April 12, 2024; U.S. Provisional Application No. 63/634,877, filed April 16, 2024; and U.S. Provisional Application No. 63/646,600, filed May 13, 2024; all of which are incorporated by reference herein.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The Sequence Listing concurrently submitted herewith as file name CX10-264WO4_ST26.xml, created on April 11, 2025, with a file size of 47,860 bytes, is part of the specification and is incorporated by reference herein.

FIELD

[0003] Described herein are methods for synthesizing oligonucleotides using an enzymatic process. Also described are systems that can be used to perform such methods.

BACKGROUND

[0004] Synthetic oligonucleotides (oligos) are significant reagents in research and medicine and are currently poised to be in greater demand. At present, the pharmaceutical industry is projected to demand hundreds to thousands of kilograms of oligonucleotides per year for active pharmaceutical ingredients (see, e.g., Andrews, Benjamin I et al. "Sustainability Challenges and Opportunities in Oligonucleotide Manufacturing." The Journal of Organic Chemistry, 2021, 86:49-61). A barrier to widescale and commercial adoption of synthetic oligos as products in multiple fields is the ability to efficiently synthesize RNA, DNA, and other polynucleotides. Current manufacturing methods rely on phosphoramidite chemistry as a means for synthesizing oligonucleotides, but the method is best suited for producing small quantities of DNA and RNA. Additionally, RNA synthesis using phosphoramidite synthesis chemistry is limited to producing short oligonucleotides of approximately 200 base pairs (Beaucage & Caruthers, Tetrahedron Lett., 1981, 22(20):1859) Despite the growing demand, optimization of the technology has only been able to achieve incremental gains in oligonucleotide length, yield and efficiency.

[0005] Phosphoramidite chemistry also requires large amounts of environmentally harmful reagents, such as acetonitrile and dichloroacetic acid, to drive conversion efficiency. For example, 1 kilogram of small interfering RNA (siRNA) may require as much as 1000 kg of acetonitrile. This results in thousands of kilograms of chemical waste per kilogram of active pharmaceutical ingredient.

SUMMARY OF THE INVENTION

[0006] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising:

elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase; removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed. In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide. In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the method further comprises separating the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase. In some aspects, the method further comprises repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety. In some aspects, at least two different types of transferases are used in separate cycles. In some aspects, the elongating occurs in a first reaction chamber. In some aspects, the removing the 3′ blocking moiety occurs in a second reaction chamber. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide

comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0007] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: in a first reaction chamber, elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber, wherein the transferase is retained in the first reaction chamber; in the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed; and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the second reaction chamber into the first reaction chamber. In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide. In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the hydrolase is retained in the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows out of the second reaction chamber. In some aspects, the method further comprises repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety. In some aspects, at least two different types of transferases are used in separate cycles. In some aspects, the first reaction chamber and the second reaction chamber are connected to each other through one or more conduits, and the elongated oligonucleotide comprising the 3′ blocking moiety and the elongated oligonucleotide without the 3′ blocking moiety flows through at least a portion of the one or more conduits. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second

reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more pumps. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more valves. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the first reaction chamber. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the second reaction chamber. In some aspects, the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the second reaction chamber. In some aspects, the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the first reaction chamber. In some aspects, the flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into the first reaction chamber comprises: flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into one or more reservoirs, and flowing the elongated oligonucleotide without the 3′ blocking moiety from the one or more reservoirs into the first reaction chamber. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking

moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0008] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising, in a plurality of reaction chambers: in at least a first reaction chamber, elongating an oligonucleotide in solution by attaching a first nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a first transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution from the at least the first reaction chamber into at least a second reaction chamber, wherein the first transferase is retained in the at least the first reaction chamber; in the at least the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed; flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the at least the second reaction chamber into at least a third reaction chamber comprising a second transferase; and in the at least the third reaction chamber, elongating the elongated oligonucleotide without the 3′ blocking moiety the solution by attaching a second nucleotide comprising a 3′ blocking moiety to the elongated oligonucleotide without the 3′ blocking moiety using the second transferase to make a further elongated oligonucleotide comprising the 3′ blocking moiety of the second nucleotide. In some aspects, the first transferase and the second transferase are different types of transferases. In some aspects, the first nucleotide and the second nucleotide are preselected types of nucleotides. In some aspects, the first nucleotide and the second nucleotide are different types of nucleotides. In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the hydrolase is retained in the at least the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows from the second reaction chamber into the at least the third reaction chamber. In some aspects, the first reaction chamber, the second reaction chamber, and the third reaction chamber are connected

through a plurality of conduits, wherein: the elongated oligonucleotide comprising the 3′ blocking moiety flows from the at least the first reaction chamber into at least a second reaction chamber through a first portion of the plurality of conduits; and the elongated oligonucleotide without the 3′ blocking moiety flows from the at least the second reaction chamber into at least a third reaction chamber through a second portion of the plurality of conduits. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more pumps. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more valves. In some aspects, the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety. In some aspects, the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution. In some aspects, the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the hydrolase is immobilized on a solid support. In some aspects, the hydrolase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the transferase is a polymerase from the DNA polymerase X family. In some aspects, the transferase is a template independent transferase. In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). In some aspects, the transferase is immobilized on a solid support. In some aspects, the transferase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, elongating produces an inorganic pyrophosphate byproduct. In some aspects, the method further comprises degrading the inorganic pyrophosphate using a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the pyrophosphatase is immobilized on a solid support. In some aspects, the pyrophosphatase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the elongating and the degrading occur within the same reaction chamber. In some aspects, the transferase and the inorganic pyrophosphatase are fused together. In some aspects, the transferase and the pyrophosphatase are immobilized on the same solid support. In some aspects, the transferase and the pyrophosphatase are immobilized on different solid supports. In some aspects, the transferase is immobilized on a solid support and the pyrophosphatase is in the solution. In some aspects, the pyrophosphatase is retained in the reaction chamber comprising the transferase, wherein the reaction chamber comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide. In some aspects, the elongating and the degrading occur within different reaction chambers. In some aspects, the method comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety and the inorganic

pyrophosphate byproduct, in the solution, from a reaction chamber comprising the transferase into a reaction chamber comprising the pyrophosphatase, followed by flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reaction chamber comprising the pyrophosphatase into a reaction chamber in which the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the first reaction chamber or the second reaction chamber is a column. In some aspects, the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a fluidized bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a fluidized bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a filter that prevents passage of hydrolase and allows passage of the oligonucleotide. In some aspects, the first reaction chamber or the second reaction chamber is a batch reaction chamber. In some aspects, the batch reaction chamber comprises an impeller. In some aspects, the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support. In some aspects, the second reaction chamber comprises a rotating bed reactor comprising hydrolase immobilized on a solid support. In some aspects, the method further comprises separating unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography. In some aspects, the liquid chromatography comprises size exclusion chromatography. In some aspects, the liquid chromatography comprises reverse phase chromatography. In some aspects, the liquid chromatography comprises ion exchange chromatography. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using tangential flow filtration. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides. In some aspects, the 3′ blocking moiety is a phosphate moiety. In some aspects, the nucleotide comprising the 3′ blocking moiety is a ribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety is a deoxyribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification. In some aspects, the 2′ modification is 2′-F or 2′-O-Me (2’-O-methyl). In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate. In some aspects, the oligonucleotide comprises a 5′ modification. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the

transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0009] Provided herein is a system for template-free synthesis of an oligonucleotide comprising: a first reaction chamber comprising a transferase; and a second reaction chamber comprising a hydrolase; wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber. In some aspects, the system further comprises a temperature regulator that controls a temperature of the solution in the system. In some aspects, the temperature regulator is configured to control the temperature of the solution in the first reaction chamber or the second reaction chamber. In some aspects, the system

further comprises one or more conduits that connects the first reaction chamber and the second reaction chamber. In some aspects, the temperature regulator is a jacketed stir tank. In some aspects, the system further comprises an in-line temperature regulator that controls a temperature of the solution in at least one of the one or more conduits. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber. In some aspects, the system comprises one or more pumps configured to flow the solution from the first reaction chamber to the second reaction chamber, and from the second reaction chamber to the first reaction chamber. In some aspects, the one or more pumps are configured to control a flow rate of the solution. In some aspects, the system further comprises a third reaction chamber comprising a second transferase, wherein the system is further configured to selectively flow said oligonucleotide in the solution from the second chamber to the third reaction chamber while retaining the hydrolase in the second reaction chamber, and flow the oligonucleotide in the solution from the third reaction chamber to the second reaction chamber while retaining the second transferase in the third reaction chamber. In some aspects, the transferase and the second transferase are different types of transferase. In some aspects, the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system. In some aspects, the hydrolase can remove a 3′ blocking moiety from the oligonucleotide in the solution. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, transferase can react a nucleotide triphosphate (NTP) comprising a 3′ blocking moiety, or an analog thereof comprising a 5′ phosphate analog, with the oligonucleotide to elongate the oligonucleotide. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof is a ribonucleotide. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof is a deoxyribonucleotide. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof comprises a 2′ modification. In some aspects, the 2′ modification of the NTP or the analog thereof is 2′-F or 2′-OMe. In some aspects, the 3′ blocking moiety of the NTP or the analog thereof is a phosphate moiety. In some aspects, the hydrolase can remove a 3′ blocking moiety from unreacted nucleotides comprising a 3′ blocking moiety in the solution. In some aspects, the unreacted nucleotides are ribonucleotides. In some aspects, the unreacted nucleotides comprise a 2′ modification. In some aspects, the 2′ modification of the unreacted nucleotides is 2′-F or 2′-OMe. In some aspects, the 3′ blocking moiety of the unreacted nucleotides is a phosphate moiety. In some aspects, the unreacted nucleotides comprise a 5′ phosphate analog. In some aspects, the 5′ phosphate analog of the unreacted nucleotides is a 5′-(α-P-thio)phosphate moiety. In some aspects, the hydrolase can remove one or more 5′ phosphate moieties and/or 5′-(α-P-thio)phosphate moieties from the unreacted nucleotides in the solution. In some aspects, the hydrolase is immobilized on a solid support. In some aspects, the hydrolase is immobilized using a covalent, electrostatic, or ionic bond.

In some aspects, the transferase is a polymerase from the DNA polymerase X family. In some aspects, the transferase is a template independent transferase. In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). In some aspects, the transferase is immobilized on a solid support. In some aspects, the transferase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the first reaction chamber further comprises a pyrophosphatase. In some aspects, the system further comprises a third reaction chamber comprising a pyrophosphatase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the pyrophosphatase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the pyrophosphatase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the pyrophosphate is retained in the third reaction chamber comprising the pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the pyrophosphatase is immobilized on a solid support. In some aspects, the pyrophosphatase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the transferase and phosphatase are fused together. In some aspects, the transferase and phosphatase are immobilized on the same solid support. In some aspects, the first reaction chamber or the second reaction chamber is a column. In some aspects, the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a fluidized bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a fluidized bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a filter that prevents passage of the hydrolase and allows passage of the oligonucleotide. In some aspects, the first reaction chamber or the second reaction chamber is a batch reaction chamber. In some aspects, the batch reaction chamber comprises an impeller. In some aspects, the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support. In some aspects, the second reaction chamber comprises a rotating bed reactor comprising the hydrolase immobilized on a solid support. In some aspects, the system further comprises a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from an oligonucleotide. In some aspects, the purification chamber comprises a column. In some aspects, the purification chamber comprises a liquid chromatography column. In some aspects, the purification chamber is a size exclusion column. In some aspects, the purification chamber is an ion exchange column. In some aspects, the purification chamber is reverse phase column. In some aspects, purification chamber is part of a tangential flow filtration system. In some aspects, the first reaction chamber or the second reaction chamber are cleanable or replaceable. In some aspects, the first reaction chamber comprises an inlet

and an outlet; the second reaction chamber comprises an inlet and an outlet; and the system further comprising a reagent reservoir comprising an inlet and an outlet; and wherein the system is configured to flow the oligonucleotide in the solution from an outlet of one of the reagent reservoir to the inlet of the first reaction chamber, from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from the outlet of the second reaction chamber to an inlet of the reagent reservoir. In some aspects, the reagent reservoir comprises an impeller. In some aspects, the reagent reservoir is replaceable or cleanable. In some aspects, the method further comprises a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the reagent reservoir. In some aspects, the system further comprises a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the first reaction chamber. In some aspects, the product reservoir comprises an impeller. In some aspects, the product reservoir is replaceable or cleanable. In some aspects, the reagent reservoir comprises the oligonucleotide and a nucleotide comprising a 3′ blocking moiety. In some aspects, the system further comprises the purification chamber, wherein the purification chamber comprises an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution, in order, from the outlet of the second reaction chamber to the inlet of the purification chamber, and from the outlet of the purification chamber to the inlet of the reagent reservoir. In some aspects, the system comprises the purification chamber, wherein the system is configured to flow the oligonucleotide, in the solution, from the outlet of the reagent reservoir to the inlet of the first reaction chamber, from the outlet of the first reaction chamber to the inlet of the second reaction chamber, from the outlet of the second reaction chamber to the inlet of the purification chamber, from the outlet of the purification chamber to the inlet of the product reservoir, and from the outlet of the product reservoir to the inlet of the reagent reservoir. In some aspects, the first reaction comprises an inlet and an outlet; the second reaction chamber comprises an inlet and an outlet; and the system further comprising a reagent reservoir comprising an inlet and an outlet, and one or more diverter valves configured to control a flow pathway of the solution; wherein the system is configured to controllably flow the oligonucleotide in the solution through the flow pathway selected from a plurality of flow pathways comprising (i) a first flow pathway comprising flow of the oligonucleotide in the solution from the outlet of the reagent reservoir to the inlet of the first reaction chamber without flowing through the second reaction chamber, and (ii) a second flow pathway comprising flow of the oligonucleotide in the solution from the outlet of the reagent reservoir to the inlet of the second reaction chamber without flowing through the first reaction chamber. In some aspects, the system is configured to automatically select the flow pathway. In some aspects, the first flow pathway comprises flow of the solution from the outlet of the first reaction chamber to the inlet of the reagent reservoir without flowing through the

second reaction chamber; and the second flow pathway comprises flow of the solution from the outlet of the second reaction chamber to the inlet of the reagent reservoir without flowing through the first reaction chamber. In some aspects, the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a third flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent reservoir to the inlet of the purification chamber without flowing through the first reaction chamber or the second reaction chamber. In some aspects, the third flow pathway further comprises flow of the solution from the outlet of the purification chamber to the inlet of the reagent reservoir without flowing through the first reaction chamber or the second reaction chamber. In some aspects, the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a fourth flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent chamber to an inlet of the first reaction chamber, from an outlet of the first reaction chamber into an inlet of the purification chamber, and from an outlet of the purification chamber into an inlet of the reagent reservoir. In some aspects, the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a fifth flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent chamber to an inlet of the second reaction chamber, from an outlet of the second reaction chamber into an inlet of the purification chamber, and from an outlet of the purification chamber into an inlet of the reagent reservoir. In some aspects, the reagent reservoir comprises an impeller. In some aspects, the transferase in the first reaction chamber is immobilized on a solid support, and the first reaction chamber comprises an inlet, an outlet, and an impeller configured to suspend the solid support comprising the transferase; and the hydrolase in the second reaction chamber is immobilized on a solid support, and the second reaction chamber comprises an inlet, an outlet, and an impeller configured to suspend the solid support comprising the hydrolase. In some aspects, the transferase in the first reaction chamber is immobilized on a solid support within a rotating bed reactor, and the first reaction chamber comprises an inlet and an outlet; and the hydrolase in the second reaction chamber is immobilized on a solid support within a rotating bed reactor, and the second reaction chamber comprises an inlet and an outlet. In some aspects, the system comprises one or more diverter valves configured to alternatively direct flow of the solution through (i) a first flow pathways comprising flow of the solution from the outlet of the first reaction chamber to an inlet of the purification chamber, and from an outlet of the purification chamber to the inlet of the first reaction chamber, or (ii) a second flow pathway comprising flow of the solution from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from an outlet of the second reaction chamber to the inlet of the first reaction chamber. In some aspects, the system further comprises a reaction chamber comprising a primase. In some aspects, the primase is immobilized on a solid support. In some aspects, the reaction chamber comprising the primase comprises a filter that prevents passage of the primase and allows passage of a 3′ -blocked donor-acceptor oligonucleotide. In some

aspects, the system comprises a degassing system or a sparging system. In some aspects, the system comprises a degassing system, and the degassing system comprises a vacuum pump. In some aspects, the system comprises the sparging system. In some aspects, the sparging system is an in-line sparging system or is configured to sparge liquids in a reservoir. In some aspects, the unit for sparging system is configured to sparge using an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, the system comprises a degasser configured to remove oxygen from the system. In some aspects, the purification chamber is configured to concentrate the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the purification chamber comprises a membrane with a molecular weight cutoff of about 500 kDa to about 5000 kDa.

[0010] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: flowing an oligonucleotide, in a solution, from a reagent reservoir into a first reaction chamber comprising a transferase; in the first reaction chamber, elongating the oligonucleotide by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using the transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the nucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber comprising a hydrolase, wherein the transferase is retained in the first reaction chamber; in the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to make an elongated oligonucleotide without the 3′ blocking moiety; and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, into the reagent reservoir. In some aspects, the method further comprises mixing the oligonucleotide and the nucleotides comprising a 3′-blocking moiety in the reservoir. In some aspects, the method further comprises flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety, in the solution, through a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety before flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety into the reagent reservoir. In some aspects, the method further comprises flowing the oligonucleotide without the 3′ blocking moiety into a product reservoir before flowing the oligonucleotide without the 3′ blocking moiety into the reagent reservoir. In some aspects, the method further comprises replacing or cleaning the reagent reservoir before flowing the elongated oligonucleotide without the 3′ blocking moiety into the reagent reservoir. In some aspects, the method repeats the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the method further comprising flowing buffer without the oligonucleotide through the system between cycles. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with

the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0011] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: (a) elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to an oligonucleotide transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety, the elongating comprising subjecting the oligonucleotide to one or more elongation flow pathway cycles comprising: flowing the oligonucleotide and nucleotides comprising a 3′ blocking moiety from a reagent reservoir to a first reaction chamber comprising a transferase, and flowing the oligonucleotide from the first reaction chamber to the reservoir; and (b) removing the 3′ blocking moiety from the oligonucleotide in the solution to make an elongated oligonucleotide without the 3′ blocking moiety, comprising subjecting the oligonucleotide to one or more deblocking flow pathway

cycles comprising: flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent reservoir to a second reaction chamber comprising a hydrolase, and flowing the elongated oligonucleotide from the second reaction chamber to the reagent reservoir. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0012] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: (a) elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to an oligonucleotide transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety, the elongating comprising subjecting the oligonucleotide to one or more elongation flow pathway cycles comprising: flowing the oligonucleotide and 3′-blocked nucleotides from a reagent

reservoir to a first reaction chamber comprising a transferase, and flowing the oligonucleotide from the first reaction chamber to the reservoir; (b) flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent reservoir to a second reaction chamber comprising a hydrolase; and (c) removing the 3′ blocking moiety from the oligonucleotide in the solution to make an elongated oligonucleotide without the 3′ blocking moiety, comprising subjecting the oligonucleotide to one or more deblocking flow pathway cycles comprising: flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the second reaction chamber to a product reservoir, and flowing the elongated oligonucleotide from the product reservoir to the second reaction chamber. In some aspects, the elongating comprises subjecting the oligonucleotide to a plurality of elongation flow path cycles. In some aspects, the removing comprises subjecting the oligonucleotide to a plurality of deblocking flow pathway cycles. In some aspects, the method comprises separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the separating comprises subjecting the oligonucleotide to one or more purification flow pathway cycles comprising: flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent reservoir or the product reservoir to a purification chamber configured to separate the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety, and flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety from the purification chamber to the reagent reservoir or the product reservoir. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography. In some aspects, the liquid chromatography comprises size exclusion chromatography. In some aspects, the liquid chromatography comprises reverse phase chromatography. In some aspects, the liquid chromatography comprises ion exchange chromatography. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using tangential flow filtration. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides. In some aspects, the method comprises repeating the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some

aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0013] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: in first reaction chamber, elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase immobilized on a solid support suspended in the solution to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber, wherein the transferase is retained in the first reaction chamber; in the second reaction chamber, removing the 3′ blocking moiety from the an elongated oligonucleotide comprising the 3′ blocking moiety in the solution using a hydrolase immobilized on a solid support suspended in the solution to make an elongated oligonucleotide without the 3′ blocking moiety; and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, into the first reaction chamber. In some aspects, the method further comprises separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety

or the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the separating comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the first reaction chamber to a purification chamber configured to separate unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the separating comprises flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber to a purification chamber configured to separate unreacted nucleotides or reaction byproducts from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography. In some aspects, the liquid chromatography comprises size exclusion chromatography. In some aspects, the liquid chromatography comprises reverse phase chromatography. In some aspects, the liquid chromatography comprises ion exchange chromatography. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using tangential flow filtration. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides. In some aspects, the method comprises repeating the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the hydrolase is retained in the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows out of the second reaction chamber. In some aspects, the first reaction chamber and the second reaction chamber are connected to each other through one or more conduits, and the elongated oligonucleotide comprising the 3′ blocking moiety and the elongated oligonucleotide without the 3′ blocking moiety flows through at least a portion of the one or more conduits. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more pumps. In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more valves. In some aspects, the nucleotide comprising the 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety. In some aspects, the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution. In some aspects, the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some

aspects, the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the hydrolase is immobilized on a solid support. In some aspects, the hydrolase is immobilized using a covalent, electrostatic, or in some aspects, the transferase is a template independent transferase. In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). In some aspects, the transferase is immobilized on a solid support. In some aspects, the transferase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the elongating produces an inorganic pyrophosphate byproduct. In some aspects, the method further comprises degrading the inorganic pyrophosphate using a pyrophosphatase. In some aspects, he pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the pyrophosphatase is immobilized on a solid support. In some aspects, the pyrophosphatase is immobilized using a covalent, electrostatic, or ionic bond. In some aspects, the elongating and the degrading occur within the same reaction chamber. In some aspects, the transferase and the inorganic pyrophosphatase are fused together. In some aspects, the transferase and the pyrophosphatase are immobilized on the same solid support. In some aspects, the transferase and the pyrophosphatase are immobilized on different solid supports. In some aspects, the transferase is immobilized on a solid support and the pyrophosphatase is in the solution. In some aspects, the pyrophosphatase is retained in the reaction chamber comprising the transferase, wherein the reaction chamber comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide. In some aspects, the elongating and the degrading occur within different reaction chambers. In some aspects, the first reaction chamber or the second reaction chamber is a column. In some aspects, the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a fluidized bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a fluidized bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a filter that prevents passage of hydrolase and allows passage of the oligonucleotide. In some aspects, the first reaction chamber or the second reaction chamber is a batch reaction chamber. In some aspects, the batch reaction chamber comprises an impeller. In some aspects, the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support. In some aspects, the second reaction chamber comprises a rotating bed reactor comprising hydrolase immobilized on a solid support. In some aspects, the 3′ blocking moiety is a phosphate moiety. In some aspects, the nucleotide comprising the 3′ blocking moiety is a ribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety is a deoxyribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification. In some aspects, the 2′ modification is 2′-F or 2′-OMe. In some aspects, the nucleotide comprising the 3′ blocking moiety

comprises a nucleoside 5′-(α-P-thio)phosphate. In some aspects, the oligonucleotide comprises a 5′ modification. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0014] Provided herein is a method of template-free synthesis of an oligonucleotide, comprising: elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase in the solution to make an elongated oligonucleotide comprising the 3′ blocking moiety; separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase; removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the

3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed. In some aspects, the method comprises separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase comprises flowing the solution through a column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide comprising the 3′ blocking moiety. In some aspects, the method comprises flowing the oligonucleotide and the transferase, in the solution, from a reagent reservoir to the column that retains the transferase, and flowing the oligonucleotide comprising the 3′ blocking moiety, in the solution, from the column that retains the transferase to the reagent reservoir. In some aspects, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety comprises adding a hydrolase to the solution after separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase. In some aspects, the method further comprises separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase comprises flowing the solution through a column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety comprises a filter that prevents passage of the hydrolase and allows passage of the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the method comprises flowing the elongated oligonucleotide without the 3′ blocking moiety and the hydrolase, in the solution, from a reagent reservoir to the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety, and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety to the reagent reservoir. In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the method comprises replacing or cleaning the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety or the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the cleaning comprises flowing a buffer through the column to elute the transferase or the hydrolase. In some aspects, the method further comprises

separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety comprises flowing the solution through a column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a size exclusion column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a reverse phase column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is an ion exchange column. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety using dialysis. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety using tangential flow filtration. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety after elongating the oligonucleotide by four or more nucleotides. In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides. In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide. In some aspects, the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety. In some aspects, the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution. In some aspects, the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the transferase is a polymerase from the DNA polymerase X family. In some aspects, the transferase is a template independent transferase. In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). In some

aspects, elongating produces an inorganic pyrophosphate byproduct. In some aspects, the method further comprises degrading the inorganic pyrophosphate using a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the transferase and the inorganic pyrophosphatase are fused together. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety further separates the pyrophosphatase from the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the 3′ blocking moiety is a phosphate moiety. In some aspects, the nucleotide comprising the 3′ blocking moiety is a ribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety is a deoxyribonucleotide. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification. In some aspects, the 2′ modification is 2′-F or 2′-OMe. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate. In some aspects, the oligonucleotide comprises a 5′ modification. In some aspects, the oligonucleotide is at least 4 nucleotides in length. In some aspects, the method comprises making the oligonucleotide prior to elongating the oligonucleotide with the transferase. In some aspects, making the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase. In some aspects, the nucleotide acceptor is a nucleotide monomer. In some aspects, the nucleotide acceptor is a nucleotide polymer. In some aspects, the nucleotide polymer is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer. In some aspects, the nucleotide donor is a nucleotide monomer. In some aspects, the nucleotide donor comprises a 3′-blocking moiety. In some aspects, the 3′-blocking moiety of the nucleotide donor is a phosphate. In some aspects, the method comprises removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor. In some aspects, the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase. In some aspects, making the oligonucleotide comprises one or more primase extension cycles, comprising: attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide; inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide; removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide; wherein each cycle uses a new nucleotide donor. In some aspects, the transferase comprises a single strand RNA ligase. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker. In some aspects, the nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combinations thereof. In some aspects, the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety or a 5′-OH. In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the

3′ blocking moiety is performed between 45 °C and 55 °C. In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

[0015] Provided herein is a system for template-free synthesis of an oligonucleotide comprising: a reagent reservoir; a first column configured to substantially separate a transferase from an oligonucleotide comprising a 3′ blocking moiety; and a second column configured to substantially separate a hydrolase from an oligonucleotide without the 3′ blocking moiety; wherein the system is configured to (i) flow a solution comprising an oligonucleotide comprising the 3′ blocking moiety and a transferase from the reagent reservoir to the first column, (ii) substantially separate the oligonucleotide comprising the 3′ blocking moiety from the transferase in the first column, (iii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety from the first column to the reagent reservoir, (iv) flow the solution comprising an oligonucleotide without the 3′ blocking moiety and a hydrolase to the second column, (v) substantially separate the oligonucleotide without the 3′ blocking moiety from the hydrolase in the second column, and (vi) flow the solution comprising the oligonucleotide without the 3′ blocking moiety from the second column to the reagent reservoir. In some aspects, the system comprises a plurality of conduits that connects the first column, the second column, and the reagent reservoir. In some aspects, the system further comprises a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety. In some aspects, the system further comprises one or more conduits that connects the purification chamber and the reagent reservoir. In some aspects, the purification chamber comprises a column. In some aspects, the purification chamber comprises a liquid chromatography column. In some aspects, the purification chamber is a size exclusion column. In some aspects, the purification chamber is an ion exchange column. In some aspects, the purification chamber is reverse phase column. In some aspects, the purification chamber is part of a tangential flow filtration system. In some aspects, the system is further configured to (vii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety from the reagent reservoir to the purification chamber, (viii) separate unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety in the purification chamber, and (ix) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety from the purification chamber to the reagent reservoir. In some aspects, the system further comprises one or more diverter valves configured to controllably flow the solution from the reagent reservoir to the first column or the second column. In some aspects, the one or more diverter valves is configured to select a flow path for the solution, wherein the flow path is selected from a plurality of flow paths comprising (i) flow of the solution from the reagent reservoir to the first

column and from the first column to the reagent reservoir and (ii) flow of the solution from the reagent reservoir to the second column and from the second column to the reagent reservoir. In some aspects, the system comprises the purification chamber, and the one or more diverter valves is further configured to controllably flow the solution from the reagent reservoir to the purification chamber. In some aspects, the plurality of flow paths further comprises (iii) flow of the solution from the reagent reservoir to the purification chamber and from the purification chamber to the reagent reservoir. In some aspects, the system further comprises one or more wash buffer reservoirs connected to the first column, the second column, or the purification chamber. In some aspects, the one or more diverter valves is configured to controllably flow wash buffer from the wash buffer reservoir to the first column, the second column, or the purification chamber. In some aspects, the one or more diverter valves is configured to select a wash buffer flow path for wash buffer in the one or more wash buffer reservoirs, wherein the flow path is selected from a plurality of flow paths comprising (i) flow of the wash buffer from the one or more wash buffer reservoirs to the first column and from the first column to a system waste outlet and (ii) flow of the wash buffer from the one or more wash buffer reservoirs to the second column and from the second column to the system waste outlet. In some aspects, the system comprises the purification chamber, and wherein the plurality of flow paths further comprises (iii) flow of the wash buffer from the one or more wash buffer reservoirs to the purification chamber and from the purification chamber to the system waste outlet. In some aspects, the system further comprises a temperature regulator that controls a temperature of the solution in the system. In some aspects, the temperature regulator is configured to control the temperature of the solution in the first column or the second column. In some aspects, the system comprises an in-line temperature regulator that controls a temperature of the solution in one or more conduits of the system. In some aspects, the temperature regulator is a jacketed stir tank. In some aspects, the system comprises one or more pumps configured to flow the solution from the reagent reservoir to the first column or from the reagent reservoir to the second column. In some aspects, the system comprises the purification chamber, and the one or more pumps are further configured to flow the solution from the reagent reservoir to the purification chamber. In some aspects, the one or more pumps are configured to control a flow rate of the solution. In some aspects, the first column is further configured to substantially separate a pyrophosphatase from the oligonucleotide comprising a 3′ blocking moiety. In some aspects, the system further comprises a third column configured to substantially separate a pyrophosphatase from the oligonucleotide comprising a 3′ blocking moiety, wherein the system is further configured to flow a solution comprising the oligonucleotide comprising the 3′ blocking moiety and the pyrophosphatase from the reagent reservoir to the third column, (ii) substantially separate the oligonucleotide comprising the 3′ blocking moiety from the pyrophosphatase in the third column, and (iii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety from the third column to the reagent reservoir. In some aspects, the first column comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the

first column comprises a resin that binds the transferase. In some aspects, the first column is an ion exchange column, an affinity column, a size exclusion column, or reverse phase column. In some aspects, the second column comprises a filter that prevents passage of the hydrolase and allows passage of the oligonucleotide. In some aspects, the second column comprises a resin that binds the transferase. In some aspects, the second column is an ion exchange column, an affinity column, a size exclusion column, or reverse phase column. In some aspects, the reagent reservoir comprises an impeller. In some aspects, the reagent reservoir comprises a reagent port. In some aspects, the first column or the second column are cleanable or replaceable. In some aspects, the system further comprises a reaction chamber comprising a primase. In some aspects, the primase is immobilized on a solid support. In some aspects, the reaction chamber comprising the primase comprises a filter that prevents passage of the primase and allows passage of a 3′ -blocked donor-acceptor oligonucleotide. In some aspects, the system comprises a degassing system or a sparging system. In some aspects, the system comprises a degassing system, and the degassing system comprises a vacuum pump. In some aspects, the system comprises the sparging system. In some aspects, the sparging system is an in-line sparging system or is configured to sparge liquids in a reservoir. In some aspects, the unit for sparging system is configured to sparge using an inert gas. In some aspects, the inert gas is argon or nitrogen. In some aspects, the system comprises a degasser configured to remove oxygen from the system. In some aspects, the purification chamber is configured to concentrate the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the purification chamber comprises a membrane with a molecular weight cutoff of about 500 kDa to about 5000 kDa.

[0016] Provided herein is a composition comprising the oligonucleotide made according to the methods described herein. In some aspects, the oligonucleotide is substantially free of depurination or depyrimidination impurities. In some aspects, the oligonucleotide is substantially free of N-3-cyanoethylthymine (CNET) impurities. In some aspects, the oligonucleotide is substantially free of N(2)-actyl-2,6-diaminopurine and/or an isobutyryl diaminopurine impurities. In some aspects, the oligonucleotide is substantially free of methylcytosine.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Various aspects of the disclosed systems and methods are set forth with particularity in the appended claims. A better understanding of the features and advantages of the disclosed systems and methods can be obtained by reference to the detailed description of illustrative aspects and the accompanying drawings.

[0018] **FIG. 1** shows a schematic of one cycle of template-free synthesis of an oligonucleotide, according to some aspects.

[0019] **FIG. 2A** shows an exemplary rotating bed reactor that may be used in a reaction chamber described herein, according to some embodiments.

[0020] **FIG. 2B** shows an exemplary reaction chamber, which is a flow-through column having a fixed bed reactor, in accordance with some embodiments. See Basso et al., *Industrial applications of immobilized enzymes – A review*, Molecular Catalysis, vol. 479, no. 110607 (2019).

[0021] **FIG. 2C** shows another exemplary reaction chamber, which is a flow-through column having a fluidized bed reactor, in accordance with some embodiments.

[0022] **FIG. 2D** shows another exemplary reaction chamber, which is a batch reaction chamber having an impeller that can stir contents of the batch reaction chamber, according to some embodiments. This type of reaction chamber may be referred to as a stirred reactor.

[0023] **FIG. 3** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container/chamber and a second container/chamber that are fluidly connected, in accordance with some embodiments. The first container/chamber comprises a transferase, and the second container/chamber comprises a hydrolase.

[0024] **FIG. 4** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container/chamber and a second container/chamber that are fluidly connected, and flow is regulated by a control valve, in accordance with some embodiments. The first container/chamber comprises a transferase, and the second container/chamber comprises a hydrolase.

[0025] **FIG. 5** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container/chamber and a second container/chamber that are fluidly connected, and the flow is regulated by a pump, in accordance with some embodiments. The first container/chamber comprises a transferase, and the second container/chamber comprises a hydrolase.

[0026] **FIG. 6** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container/chamber and a second container/chamber that are fluidly connected to each other, in accordance with some embodiments. The flow is regulated by control valves and pumps. The first container/chamber comprises a transferase, and the second container/chamber comprises a hydrolase.

[0027] **FIG. 7** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container/chamber and a second container/chamber that are fluidly connected to each other, in accordance with some embodiments. The flow is regulated by control valves and pumps. The first container/chamber comprises a transferase, and the second container/chamber comprises a hydrolase. The system comprises a reaction chamber 3, which can comprise an enzyme such as another transferase.

[0028] **FIG. 8** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container and a second container that are fluidly connected to each other, in accordance with some embodiments. The flow is regulated by control valves and pumps. The first

container comprises a transferase, and the second container comprises a hydrolase. The system comprises a reagent reservoir that can also act as a product reservoir.

[0029] **FIG. 9** shows an exemplary system for template-free synthesis of an oligonucleotide, wherein the system comprises a first container and a second container that are fluidly connected to each other, in accordance with some embodiments. The flow is regulated by control valves and pumps. The first container comprises a transferase, and the second container comprises a hydrolase. The system comprises a reagent reservoir that can also act as a product reservoir. One or more reagent reservoirs can be connected upstream of the primary reagent reservoir.

[0030] **FIG. 10** shows an exemplary system for template-free synthesis of an oligonucleotide using chambers, such as in-line reaction chambers with enzymes being retained in the reaction chambers as the synthesized oligonucleotide can flow out of the reaction chambers, in accordance with some embodiments. The oligonucleotide is transferred through the system for extension and cycling using linear flow.

[0031] **FIG. 11** shows an exemplary system for template-free synthesis of an oligonucleotide using reaction chambers such as in-line columns, in accordance with some embodiments. In this particular schematic, the enzymes are immobilized on a solid support, such as a fluidized bed or a fixed bed. The oligonucleotide is transferred through the system for extension and cycling using flow controlled by a pump.

[0032] **FIG. 12** shows an exemplary system for template-free synthesis of an oligonucleotide using reaction chambers such as stirred reactor, in accordance with some embodiments. The oligonucleotide is transferred through the system for extension and cycling using flow controlled by a pump.

[0033] **FIG. 13** shows an exemplary system for template-free synthesis of an oligonucleotide using a reaction chamber such as a stirred reactor, in accordance with some embodiments. Purification chambers such as in line columns can be used for removing enzyme. The oligonucleotide is transferred through the system for extension and cycling using flow controlled by a pump.

[0034] **FIG. 14** shows an exemplary system for template-free synthesis of an oligonucleotide using reaction chambers such as columns, in accordance with some embodiments. Desalting chambers can be used for separating or removing salts, reagents and other small molecule byproducts from the oligonucleotide. The oligonucleotide is transferred through the system for extension and cycling using flow controlled by a pump.

[0035] **FIG. 15** shows an exemplary system for template-free synthesis of an oligonucleotide using reaction chambers such as columns, in accordance with some embodiments. Desalting chambers can be used for separating or removing salts, reagents and other small molecule byproducts from the

oligonucleotide. The oligonucleotide is transferred through the system for extension and cycling using flow controlled by a pump.

[0036] **FIG. 16** shows an impurity profile of an oligonucleotide synthesized by template-free synthesis.

DETAILED DESCRIPTION

[0037] The present disclosure relates generally to methods of template-free synthesis of an oligonucleotide, as well as systems related thereto. The systems can be embodied by any of the system designs and/or combination of the system designs as described herein. More specifically, the present disclosure relates to the discovery of systems and methods that enable template-free synthesis, and especially commercial scale template-free synthesis, of oligonucleotides in an efficient and economical manner. Also provided herein are methods that use these systems described herein.

[0038] Oligonucleotides were previously known to be synthesized in a manner that requires a large, upfront investment in reagents, such organic solvents, to drive the large-scale production at the expense of producing large amounts of chemical waste is also costly to dispose. Additionally, current enzymatic and or chemical methods for oligonucleotide synthesis do not guarantee commercially viable yields. Current methods produce oligonucleotides at lower scales because the process of elongation and extension compromise product quality through degradation, or product yield is reduced because it must be recovered from contaminants and other byproducts that are generated from the synthesis process. Additionally, current synthesis techniques that aim to increase purity, increase oligonucleotide length and/or reduce incorporation error or degradation require manual intervention at the completion of each cycle, and thus are optimized to be operated under batch production conditions, where reagents are discarded due to contaminating solvents or otherwise rendered inoperable for further use.

[0039] For enzymatic synthesis of oligonucleotides, one such reagent that is limited in quantity and cost prohibitive is enzyme. The quantity of enzyme required using existing systems and methods for template independent enzymatic oligonucleotide synthesis would be cost prohibitive at a commercial scale. The inventors have discovered systems and methods that retain the enzyme in their reaction chamber, while the oligonucleotide (e.g., the substrate oligonucleotide) is flowed in solution between chambers and reservoirs. Retaining enzyme in the reaction chamber permits for re-use, decreasing the total quantity of enzyme required for synthesis of oligonucleotide of a predetermined sequence.

[0040] The oligonucleotides that can be synthesized using these systems and methods are of a pre-determined sequence can be made with high target yields and accuracy in solution (e.g., under flow manufacturing conditions), requiring little user intervention between iterative extension cycles and enhancing reagent efficiency, therefore making the oligonucleotides commercially viable as a reagent or active pharmaceutical ingredient. These systems and methods also reduce reagent use and cost and

remove the barriers that inhibit large scale oligonucleotide synthesis. Moreover, the inventors have surprisingly discovered that systems and methods permit large scale production of pre-determined oligonucleotides lengths that are longer compared to what is presently achievable by current synthesis techniques in an economic manner.

[0041] As such, the systems and methods disclosed herein offer a substantial improvement over enzyme mediated oligonucleotide synthesis and chemical methods of oligonucleotide synthesis.

[0042] Disclosed herein are methods that provide for controlled elongation of an oligonucleotide in solution is achieved using a nucleotide comprising a 3′ blocking moiety and a transferase, yielding an elongated oligonucleotide comprising a 3′ blocking moiety. The 3′ blocking moiety prevents additional addition of nucleotides because it sterically hinders further elongation of the oligonucleotide chain by the transferase. The oligonucleotide comprising the 3′ is retained in solution, then is then separated from the enzyme, and deblocked by removing a 3′ blocking moiety to produce an elongated oligonucleotide. The deblocking is also conducted with the oligonucleotide in solution. The methods described herein describe a method of template-free synthesis of an oligonucleotide, where the oligonucleotide remains in solution (e.g., the liquid phase). As such, the discovery of the systems and the methods that use the systems as described herein enable efficient and economical template-free synthesis of an oligonucleotide through batch or flow production methods.

[0043] Disclosed herein are also systems and methods that provide for elongating the oligonucleotide in a first reaction chamber, separating the elongated oligonucleotide comprising the 3′ blocking moiety by flowing it to a second reaction chamber, and removing the 3′ blocking moiety in a second reaction chamber. Separating the elongated oligonucleotide comprising the 3′ blocking moiety is achieved by retaining transferase in the first reaction chamber. After removal of the 3′ blocking moiety from the elongated oligonucleotide, the elongated oligonucleotide is flowed from the second reaction chamber back to the first reaction chamber to re-initiate the cycle of elongation and 3′ blocking moiety removal (e.g., an exemplary cycle as in **FIG. 1**) to further elongate the oligonucleotide by another nucleotide. Because the nucleic acid (e.g., oligonucleotide, elongated oligonucleotide comprising a 3′ blocking moiety, elongated oligonucleotide) remains in solution, the nucleic acid can be moved through flowing the solution through any of the systems as described herein to perform the any of the methods of template-free synthesis disclosed herein. In some aspects, the systems and methods described herein comprise at least a third reaction chamber, wherein the elongated oligonucleotide can be further elongated by a second transferase to produce a further elongated oligonucleotide comprising the 3′ blocking moiety of the second nucleotide.

[0044] Disclosed herein are also methods and systems that can be used for methods of template-free synthesis of an oligonucleotide by elongating an oligonucleotide in solution using a transferase in solution, then separating the elongated oligonucleotide from the transferase, and removing the 3′

blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety.

[0045] Disclosed herein are also systems and methods for template-free synthesis of an oligonucleotide comprising a first reaction chamber that comprises a transferase, and a second reaction chamber that comprises a hydrolase. These reaction chambers are fluidly connected such that an oligonucleotide (such as an oligonucleotide in solution) can be flowed between the chambers. In the first chamber, an oligonucleotide can be elongated by a transferase, which attaches a 3′ blocking moiety to the oligonucleotide, producing an elongated oligonucleotide comprising a 3′ blocking moiety. The elongated oligonucleotide comprising a 3′ blocking moiety is then flowed to a second reaction chamber, while the transferase is retained in the first chamber. The elongated oligonucleotide comprising a 3′ blocking moiety enters the second chamber, which comprises a hydrolase. The hydrolase removes the 3′ blocking moiety from the elongated oligonucleotide comprising a 3′ blocking moiety, producing an elongated oligonucleotide. The elongated oligonucleotide can then be flowed from the second chamber back to the first chamber, where it can be further elongated by the transferase that was retained in the first chamber. This cycle can be iterated any number of times with the addition of a predetermined nucleotide comprising a 3′ blocking moiety to achieve an oligonucleotide of a predetermined sequence.

[0046] Disclosed herein are also systems that further comprise additional modules that such as reservoirs. In some aspects, the reservoir is a reagent reservoir that holds additional reagents necessary for the methods described herein, such as buffers, cofactors, and predetermined nucleotides comprising a 3′ blocking moiety. In some aspects, the reservoir is a product reservoir that holds or collects product (e.g., an elongated oligonucleotide) after the 3′ moiety is removed from the elongated oligonucleotide comprising a 3′ moiety. In some aspects, the product reservoir is fluidly connected to the first reaction chamber or the reagent reservoir. In some aspects, the reagent reservoir also acts as a product reservoir.

[0047] Disclosed herein are systems that are configured to separate the transferase from the oligonucleotide comprising a 3′ blocking moiety; and a second column configured to substantially separate a hydrolase from an oligonucleotide without the 3′ blocking moiety. The system is further configured with a reagent reservoir, wherein the solution comprising the oligonucleotides are flowed into said reagent reservoir to substantially separate the nucleic acid (e.g., an elongated oligonucleotide comprising a 3′ blocking moiety or an elongated oligonucleotide) from the enzyme (e.g., a hydrolase or a transferase) prior to being flowed to the next reaction chamber.

[0048] Disclosed herein are also systems that further comprise additional modules that control the flow or regulate system conditions. In some aspects, the systems comprise one or more reaction pumps. In some aspects, the systems comprise one or more valves that selectively control a pathway

of the solution in the system. These optional features may be incorporated into the systems described herein to permit enzymatic synthesis.

[0049] These systems as described herein can be automated or configured to flow the substrate oligonucleotide in solution between chambers while retaining the enzymes (e.g., the hydrolase or transferase) in their respective reaction chambers such that they can be reused in the next iterative cycle of oligonucleotide elongation and removal of the 3′ blocking moiety from the elongated oligonucleotide. The systems and methods described herein are also configured retain and enable the re-use of the hydrolase, transferase, and/or pyrophosphatase in the downstream cycles of template-free synthesis of the oligonucleotide. In certain aspects, the systems described herein can be automated or partially automated. In certain aspects, these systems can be embodied by the systems that are described in further detail herein. In certain aspects, these systems described herein can further comprise optional features such as one or more temperature regulators, thermometers, valves, pumps, degassers (such as vacuum pumps), spargers (e.g., an in-line sparger and/or a sparger in a reservoir), and flow regulators.

Definitions

[0050] 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.

[0051] 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.

[0052] It is to be understood that where descriptions of various aspects 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 aspects 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.

[0053] "Substantially separated" as used herein refers to dividing, excluding, partitioning, or removing 95% or more of a macromolecular species by percent weight. For example, an enzyme (e.g., transferase, hydrolase, pyrophosphatase, etc.) is considered substantially separated from an oligonucleotide if not more than 5% of the starting quantity is included in the solution after the enzyme has been substantially separated from the oligonucleotide (such as an elongated oligonucleotide comprising the 3′ blocking moiety).

[0054] "Substantially free" as used herein refers to a composition that contains less than 5% or less of the identified component (e.g., impurity) by percent weight. For example, a composition that is substantially free of an identified impurity contains 5% or less of that impurity.

[0055] As used herein, "nucleotide triphosphate" or "NTP" as used herein refers to a nucleoside with three 5′ phosphate groups (or 5′ phosphate group analogs) but does not exclude other nucleotides comprising additional phosphate (or phosphate analog) moieties. NTPs may also comprise additional modifications, such as on the 3′ position of the ribose sugar (e.g., a 3′ phosphate group such that the NTP comprises a 3′ phosphate group), on the 2′-position of the ribose sugar (OMe, F, H, MOE), locked and glycine NTPs. NTPs may also be also substituted at any of the 5′ phosphate groups to create NTP analogs. For example, a non-bridging oxygen on the α-phosphate may be substituted with a sulfur to make nucleoside 5′-(α-P-thio)triphosphates. NTPs may also be modified to remove a nucleobase to generate an abasic nucleotide.

[0056] As used herein, "nucleotide analog" or "NTP analog" refers to a modified NTP that has been chemically modified and has biosimilar properties and functionality to naturally occurring NTPs. NTP analogs are structurally similar to NTPs. For example, an analog of a NTP can comprise a nucleoside 5′-(α-P-thio)triphosphate moiety.

[0057] 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′-flouro, 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 phosphothiolated linkages. 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 aspects, 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.

[0058] As used herein, "template-free 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-free synthesis refers to an iterative process, whereby, successive nucleotides are added to a growing oligo or nucleotide chain or oligonucleotide substrate.

[0059] "Substrate" or "reagent" in the context of an enzyme mediated reaction refers to the molecule acted on by the enzyme (e.g., a transferase or a hydrolase). For example, a transferase used in the systems and methods disclosed herein act on a substrate (e.g., an oligonucleotide). In another example, a hydrolase used in the systems and methods disclosed herein act on a substrate (e.g., an elongated oligonucleotide comprising a 3′ blocking moiety or an unreacted nucleotide such as a nucleotide comprising a 3′ blocking moiety). In another example, a pyrophosphatase used in the systems and methods disclosed herein act on a substrate (e.g., inorganic pyrophosphate).

[0060] "Product" in the context of an enzyme mediated reaction refers to the molecule resulting from the action of the enzyme. In some aspects, the product is the target molecule. For example, an exemplary product for a transferase used in the systems and methods described herein is an elongated oligonucleotide comprising a 3′ blocking moiety.

[0061] "Byproduct" in the context of an enzyme mediated reaction is a secondary product that is generated in addition to the product resulting from the action of the enzyme. For example, byproducts can include, but are not limited to, nucleosides, NDPs, residual NDPs comprising a 3′ blocking moiety, NMP, residual NMPs comprising a 3′ blocking moiety, phosphate, and residual pyrophosphate.

[0062] "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. A phosphate group or chain may be modified, as further described herein.

[0063] "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).

[0064] As used herein, where the description refers to "flowing," for example without limitation, flowing of reaction solution from a reaction chamber to another reaction chamber, or a reaction chamber to a reservoir, it may also described as being in "fluid communication" between the recited elements (e.g., reaction chamber, reservoir, etc.) through conduits, such as tubes or channels. For example, a reaction chamber in fluid communication with a reservoir allows for flowing of reaction

solution between the reaction chamber and the reservoir such as through a conduit. In some embodiments, the flow of solution between the elements can be regulated, such as by valves or diverters, as described herein.

[0065] 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.

[0066] 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.

[0067] 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.

[0068] The entire disclosure of each patent, patent application, and publication (including patent publication or non-patent publication) referred in this application are hereby incorporated herein by reference for all purposes. To the extent that any reference incorporated by reference conflicts with the instant disclosure, the instant disclosure shall control.

Nucleotides and Nucleotide Analogs

[0069] The present disclosure provides systems and methods that use nucleotides and/or nucleotide analogs such as modified nucleotides. A nucleotide comprises a sugar moiety, which can be further modified at different positions. For example, modified nucleotides contemplated include nucleotides

with blocking groups on the 3′ positions of the sugar (e.g., nucleotides comprising a 3′ blocking moiety), as well as nucleotides with modified bases or thiol derivates for the formation of more stable oligonucleotide phosphorothioate backbone bonds. 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 transferase from adding additional nucleotides to the oligonucleotides. 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.

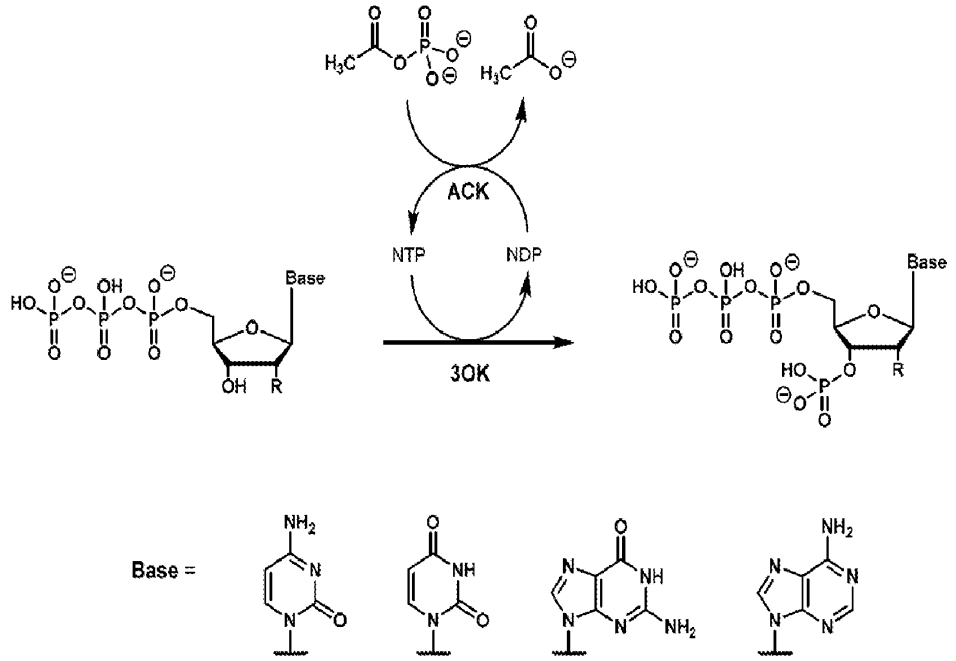
Nucleotides comprising a 3′ blocking moiety

[0070] The systems and methods provided herein can be used to elongate an oligonucleotide in solution (e.g., in a liquid) by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide, for example, by using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety. The 3′ blocking moiety can then be removed from the elongated oligonucleotide comprising the 3′ blocking moiety, producing an elongated oligonucleotide.

[0071] In some aspects, the nucleotide comprising a 3′ blocking moiety may comprise a nucleotide triphosphates (NTP). The NTP further comprises a 3′ blocking moiety (e.g., a blocking moiety on the 3′ position of the sugar of the nucleotide). As used herein, a NTP does not exclude the inclusion of other phosphates (e.g., phosphates introduced in the 3′ blocking moiety).

[0072] In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a nucleoside tetraphosphate. NTPs with a phosphate group at the 3′ position of the sugar (nucleoside tetraphosphates, pppNps or otherwise known herein as "NQPs"), with or without additional modifications to the nucleobase, sugar, and/or phosphate chain, are useful for the systems and methods of template-free synthesis as provided herein. An exemplary process of converting a NTP into a NTP with a phosphate group at the 3′ position of the sugar (e.g., a NQP) is depicted in **Scheme 1**.

Scheme 1



[0073] As depicted in **Scheme 1**, the NTP is converted by a 3′O-kinase to a nucleoside tetraphosphate (NQP or pppNp) with the fourth phosphate group at the 3′ position of the sugar, thereby generating a nucleotide comprising a 3′ blocking moiety. The group R at the 2′ position of the sugar ("2′-R group") may be an atom or group selected from H, OH, OCH_3, $OCH_2CH_2OCH_3$, F, and CO_2R' (where R' is any alkyl or aryl), or another atom or chemical group. Additionally, the sugar may have other modifications at other positions. The nucleobase may be a uridine, thymine, cytosine, adenine, guanine or another nucleobase known to those skilled in the art. Although not depicted in Scheme 1, the nucleotide comprising a 3′ blocking moiety may also have modifications of the nucleobase or of the 5′ phosphate chain.

[0074] In some aspects, the nucleotide comprising a 3′ blocking moiety may comprise one or more modifications.

[0075] In some aspects, the nucleotide comprising a 3′ blocking moiety may comprise ribonucleosides, deoxyribonucleosides, dideoxynucleosides, or modified nucleosides.

[0076] In some aspects, the nucleotide comprising a 3′ blocking moiety may comprise one or more modifications to the sugar. In some aspects, nucleotide comprising a 3′ blocking moiety may comprise one or more modifications to the nucleobase.

[0077] In some aspects, the nucleotide comprising a 3′ blocking moiety has at the 2′-position of the sugar moiety a H or OH.

[0078] In some aspects, the nucleobase of the nucleotide comprising a 3′ blocking moiety is adenine, cytosine, guanine, thymine, uracil, xanthine, hypoxanthine, 2,6-diaminopurine, purine, 6,8-diaminopurine, 5-methylcytosine (m5C), 2-thiouridine, pseudouridine, dihydrouridine, inosine, or 7-methylguanosine (m7G).

[0079] In some aspects, the nucleotide comprising a 3′ blocking moiety has a nitrogenous base that is adenosine (A), guanosine (G), uridine (U), cytidine (C), or thymidine (T), and wherein the nucleoside has at the 2′-position of the sugar moiety an OH. In some aspects, the nucleotide comprising a 3′ blocking moiety is a ribonucleotide.

[0080] In some aspects, the nucleotide comprising a 3′ blocking moiety has a nitrogenous base that is adenosine (A), guanosine (G), uridine (U), cytidine (C), or thymidine (T), and wherein the nucleoside has at the 2′-position of the sugar moiety a H. In some aspects, the nucleotide comprising a 3′ blocking moiety is a deoxyribonucleotide.

[0081] In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a modified nucleoside. In some aspects, the nucleoside has a modified sugar moiety or modified nucleobase, or combination thereof.

[0082] In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a modified 2′-position of the sugar moiety (e.g., a 2′ modification). In some aspects, the 2′ modification is halo, 2′-O-R', or 2′-O-COR', where R' is an alkyl, alkyloxyalkyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, arylalkyl, or heteroarylalkyl. In some aspects, R' is a C1-C4alkyl. In some aspects, the modified 2′-position is a 2′-O-R', wherein in R' is alkyloxyalkyl, alkylamine, cyanoalkyl, or -C(O)-alkyl. In some aspects, the 2′-position of the sugar moiety of the nucleoside substrate is -O-R', wherein R' is -CH3 or -CH2CH3 or -CH2CH2OCH3. In some aspects, the modified 2′-position is 2′-O-(2-methoxyethyl), 2′-O-allyl, 2′-O-propargyl, 2′-O-ethylamine, 2′-O-cyanoethyl, or 2-O-acetate ester. In some aspects, the 2′-position of the sugar moiety is halo. In some aspects, the 2′-position of the sugar moiety is F (i.e., 2′-F) or Br (i.e., 2′-Br). In some aspects, the 2′ modification is 2′-F. In some aspects, the 2′ modification is 2′-OMe.

[0083] In some aspects, the nucleotide comprising a 3′ blocking moiety may comprise an α-thiophosphate or dithiophosphate or other modification to the 5′ phosphate chain. In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate. A nucleotide that comprises a nucleoside 5′-(α-P-thio)phosphate is also known as an α-phosphate modified nucleotides (e.g., dNTPαS and NTPαS) and are presently used to prepare phosphorothiolate DNAs and RNAs, respectively. A phosphorothiolate bond introduces a sulfur atom in place of a non-briding oxygen in the phosphate backbone. Oligonucleotides comprising phosphorothiolate bonds are more resistant to degradation by nucleases. The present systems and methods as disclosed herein can

utilize nucleotides comprising a 3′ blocking moiety, and further comprising a 5 ′-(α-P-thio)phosphate to elongate an oligonucleotide.

[0084] In some aspects, nucleotide comprising a 3′ blocking moiety comprises a modified nucleobase. In some aspects, the modified nucleobase of the nucleoside substrate is 5-bromo-uracil, 5-iodo-uracil, 6-mCEPh-purine, 6-phenylpyrrolocytidine, N2-alkyl 8-oxoguanosine, difluorotoluene, difluorobenzene, dichlorobenzene, imidazole, or benzimidazole.

[0085] In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a nucleobase that is, among others, 5-methylcytosine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5- alkylcytidines, 5-alkyluridines, 5-halouridines, 6-azapyrimidines, 6- alkylpyrimidines, 5-propynyl-uracil, 2-thio-5-propynyl-uracil, quesosine, 2-thiouridine, 4-thiouridine, 4-acetylcytidine, 5- (carboxyhydroxymethyl)uridine, 5-carboxymethylaminomethyl-2-thiouridine, 5- carboxymethylaminomethyluridine, -D-galactosylqueosine, 1-methyladenosine, 1- methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2- methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethy1-2- thiouridine, 5-methylaminomethy luridine, 5-methylcarbon ylmethyluridine, 5- methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, -D- mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, N1-methyl-adenine, N6-methyl- adenine, 8'-azido-adenine, N,N-dimethy1-adenosine, aminoally1-adenosine, 5′-methy1-uridine, pseudouridine, N1-methy1-pseudouridine, 5′-hydroxy-methy1-uridine, 2′-thio-uridine, 4'-thio- uridine, hypoxanthine, xanthine, 5′-methyl-cytidine, 5′-hydroxy-methyl-cytidine, 6'-thio- guanine, or N7-methyl-guanine.

[0086] In some aspects, the nucleotide comprising a 3′ blocking moiety comprises a noncanonical nucleobase, a removable tag, a cleavable linker, or a radio, a photo, and/or a chemical sensor.

[0087] In some aspects, the nucleotide comprising a 3′ blocking moiety further comprises a conjugate moiety. In some aspects, the conjugate moiety is on the nucleobase of the nucleotide. In some aspects, the nucleotide comprising a 3′ blocking moiety further comprises a 2′ conjugate moiety. In some aspects, the 2′ conjugate moiety is a ligand. The conjugate moiety (i.e., non-nucleotide moiety) includes, among others, carbohydrates (e.g. GalNAc), lipids, sterols, drug substances, hormones, polymers (e.g., polyethylene glycol, etc.), proteins, peptides, toxins (e.g. bacterial toxins, etc.), vitamins (e.g., folate, tocopherol, retinoic acid, etc.), or combinations thereof. In some embodiments, the conjugate moiety is used to affect the pharmacokinetics of the oligonucleotide and/or oligonucleotide cell targeting.

[0088] In some embodiments, the conjugate moiety can be attached to the 5'-terminal nucleotide, the 3'-terminal nucleotide, or in a polynucleotide or oligonucleotide an internal nucleotide. In some embodiments, the conjugate moiety is attached the 2'-position of the sugar moiety of a nucleoside, for

example, to the 2'-OH. In some embodiments, the conjugate moiety is attached to the 3'-position of the sugar moiety of the nucleoside, for example 3'-OH. In some embodiments, the conjugate moiety is attached to the nucleobase, as discussed above (see, e.g., Biscans et al., Nucleic Acids Res. 2019 Feb 20; 47(3): 1082–1096). In some embodiments, the conjugate moiety is attached directly or attached using a linker.

[0089] In some embodiments, the conjugate moiety comprises a C_6-C_{22} alkyl, C_{6-22} alkenyl, or C_6-C_{22} alkynyl. In some embodiments, the conjugate moiety comprises a C_6-alkyl, C_7-alkyl, C_8-alkyl, C_9-alkyl, C_{10}-alkyl, C_{11}-alkyl, C_{12}-alkyl, C_{13}-alkyl, C_{14}-alkyl, C_{15}-alkyl, C_{16}-alkyl, C_{17}-alkyl, C_{18}-alkyl, C_{19}-alkyl, C_{20}-alkyl, C_{21}-alkyl, or C_{22}-alkyl. In some embodiments, the conjugate moiety comprises a C_6 alkenyl, C_7 alkenyl, C_8 alkenyl C_9 alkenyl, C_{10} alkenyl, C_{11}-alkenyl, C_{12}-alkenyl, C_{13}-alkenyl, C_{14}-alkenyl, C_{15}-alkenyl, C_{16}-alkenyl, C_{17}-alkenyl, C_{18}-alkenyl, C_{19}-alkenyl, C_{20}-alkenyl, C_{21}-alkenyl, or C_{22}-alkenyl. In some embodiments, the conjugate moiety comprises a C_6 alkynyl, C_7 alkynyl, C_8 alkynyl, C_9 alkynyl, C_{10} alkynyl, C_{11}-alkynyl, C_{12}-alkynyl, C_{13}-alkynyl, C_{14}-alkynyl, C_{15}-alkynyl, C_{16}-alkynyl, C_{17}-alkynyl, C_{18}-alkynyl, C_{19}-alkynyl, C_{20}-alkynyl, C_{21}-alkynyl, or C_{22}-alkynyl.

[0090] In some embodiments, the conjugate moiety comprises a heteroalkyl, heteroalkenyl, or heteroalkynyl. In some embodiments, the heteroalkyl, heteroalkenyl or heteroalkynyl has one or more carbon atoms replaced with a heteroatom, such as O, S, or N.

[0091] In some embodiments, the conjugate moiety comprises a cycloalkyl or heterocycloalkyl group. In some embodiments, the cycloalkyl includes, by way of example and not limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, and cycloheptyl. In some embodiments, the heterocycloalkyl includes, among others, 1-(1,2,5,6-tetrahydropyridine, 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, and 2-piperazinyl.

[0092] In some embodiments, the conjugate moiety comprises an aryl or heteroaryl moiety. In some embodiments, the aryl group includes, by way of example and not limitation, phenyl, naphthyl, indenyl, biphenyl, phenanthrenyl, naphthacenyl, anthracenyl, fluorenyl, indenyl, and azulenyl. In some embodiments, a heteroaryl group includes, among others, pyridyl, furanyl, thienyl, pynolyl, oxazolyl, oxadiazolyl, imidazolyl ihiazolyl, isoxazolyl, quinolinyl, pyrazolyl, isoihiazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl, isoquinolinyl, and indazolyl.

[0093] In some embodiments, the conjugate moiety comprises a cycloalkylalkyl-, heterocycloalkylalkyl-, arylalkyl-, heteroarylalkyl-, cycloalkylheteroalkyl-heterocycloalkylheteroalkyl-, arylheteroalkyl-, heteroarylheteroalkyl-, cycloalkylalkenyl-, heterocycloalkylalkenyl-, arylalkenyl-, heteroarylalkenyl-, cycloalkylheteroalkenyl-heterocycloalkylheteroalkenyl-, arylheteroalkenyl-, or heteroarylheteroalkenyl- groups.

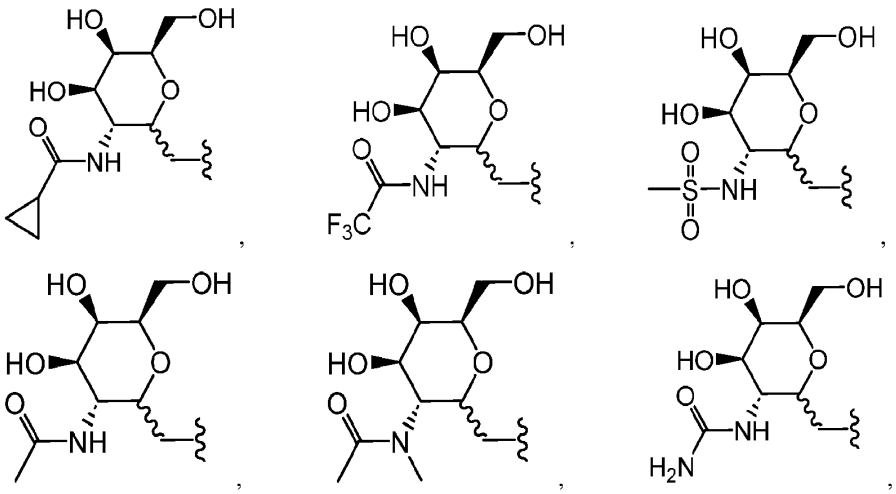
[0094] In some embodiments, the conjugate moiety comprises a lipid or lipophilic moiety, for example a fatty acid. In some embodiments, the fatty acid comprises a saturated fatty acid, unsaturated fatty acid, or a polyunsaturated fatty acid. In some embodiments, the fatty acid comprises caprylic acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, oleic acid, elaidic acid, cis-vaccenic acid, trans-vaccenic acid, linoleic acid, alpha-linoleic acid, gamma-linoleic acid, arachidonic acid, eicosapentaenoic acid, decanoic acid, docosahexaenoic acid (DHA), and docosanoic acid (DCA) conjugate moieties (see, e.g., Kubo et al., ACS Chem. Biol., 2021, 16, 150−164; see also, WO2024/040041; incorporated herein by reference).

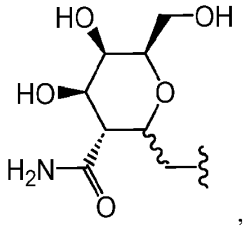
[0095] In some embodiments, the conjugate moiety comprises a sterol. In some embodiments, the sterol comprises cholesterol, alpha-cholesterol, cholesterol ester (e.g., cholesteryl palmitate, etc.), cholesterol sulfate, phytosterol, cholic acid, or lithocholic acid.

[0096] In some embodiments, the conjugate moiety comprises a vitamin or vitamin derivative, including, by way of example and not limitation, folate, tocopherol, retinoic acid, vitamin D, and the like (see, e.g., US Patent No. 9789197).

[0097] In some embodiments, the conjugate moiety comprises a phospholipid. In some embodiments, the phospholipid comprises phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, or a sphingolipid.

[0098] In some embodiments, the conjugate moiety comprises a carbohydrate, particularly a carbohydrate moiety acting as a ligand for a cellular receptor for cellular targeting of the oligonucleotide. In some embodiments, the carbohydrate moiety comprises galactose or galactose derivatives. In some embodiments, the carbohydrate moiety is attached to the nucleoside via a linker. In some embodiments, carbohydrates moiety include the following:





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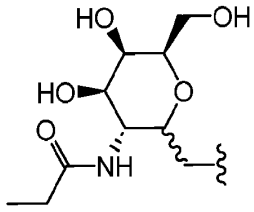

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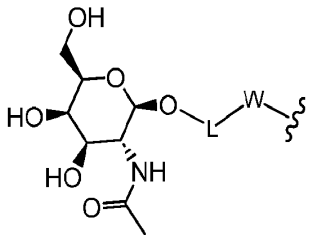
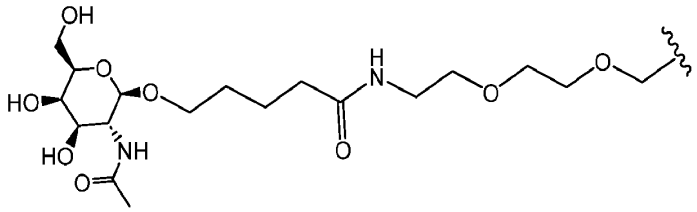
[0099] In some embodiments, the conjugate moiety is an N-acetylgalactosamine (GalNAc) conjugate moiety. In some embodiments, the oligonucleotide acceptor and/or nucleotide donor may be conjugated to at least one conjugate moiety comprising at least one N-acetylgalactosamine (GalNAc) moiety. In some embodiments, the conjugate moiety is monovalent, divalent, trivalent or tetravalent, GalNAc.

[0100] In some embodiments, the GalNAc moiety has the following structure,

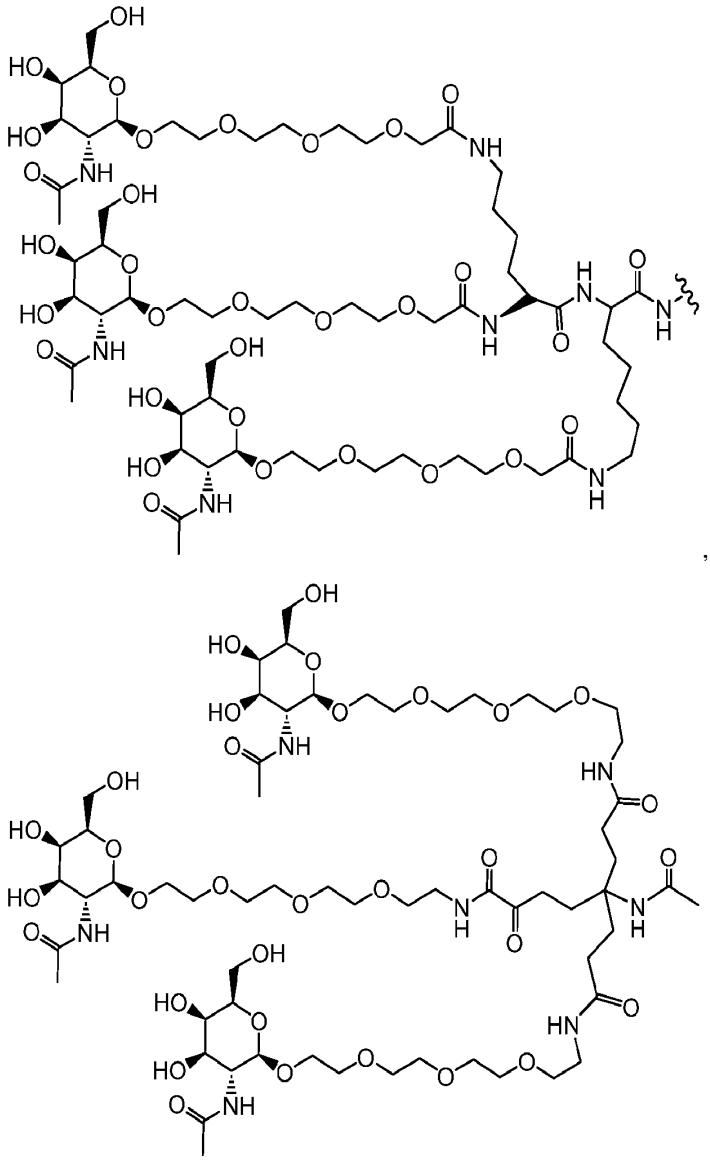


[0101] where L is a linker, and W is a heteroatom, such as O or S. In some embodiments, the W is the 2'-OH of the sugar moiety of a nucleoside. An exemplary monovalent GalNAc moiety is



[0102] wherein the monovalent GalNAc is attached via the linker to the 2'-position of a nucleoside, such as adenine or guanine. These conjugate moieties can be present in contiguous nucleotides in a polynucleotide or oligonucleotide (see, e.g., WO2024/040041).

[0103] In some embodiments, the conjugate moiety is a trivalent GalNAc. Tri-valent N-acetylgalactosamine conjugate moieties are described in, for example, International patent publication WO 2014/076196, WO 2014/207232 and WO 2014/179620. "Trivalent GalNAc" refers to a residue comprising three N-acetylgalactosamine moieties, typically attached via a linker. An exemplary trivalent GalNAc conjugate moieties are depicted below:



[0104] In some embodiments, the conjugate moiety comprises a reporter molecule. Example of reporter molecules include, among others, fluorescent moieties, such as fluorescein and fluorescein dyes (e.g., fluorescein isothiocyanine or FITC, naphthofluorescein, 4′,5′-dichloro-2′,7′-dimethoxy-fluorescein, 6-carboxyfluorescein or FAM), carbocyanine, merocyanine, styryl dyes, oxonol dyes, phycoerythrin, erythrosin, eosin, rhodamine dyes (e.g., carboxytetramethylrhodamine or TAMRA, carboxyrhodamine 6G, carboxy-X-rhodamine (ROX), lissamine rhodamine B, rhodamine 6G, rhodamine Green, rhodamine Red, tetramethylrhodamine or TMR), coumarin and coumarin dyes (e.g., methoxycoumarin, dialkylaminocoumarin, hydroxycoumarin and aminomethylcoumarin or AMCA), Oregon Green Dyes (e.g., Oregon Green 488, Oregon Green 500, Oregon Green 514), Texas

Red, Texas Red-X, Spectrum Red™, Spectrum Green™, cyanine dyes (e.g., Cy-3™, Cy-5™, Cy-3.5™, Cy-5.5™), Alexa Fluor dyes (e.g., Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 660 and Alexa Fluor 680), BODIPY dyes (e.g., BODIPY FL, BODIPY R6G, BODIPY TMR, BODIPY TR, BODIPY 530/550, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY 630/650, BODIPY 650/665), IRDyes (e.g., IRD40, IRD 700, IRD 800). (See, e.g., "The Handbook of Fluorescent Probes and Research Products", 9th Ed., R.P. Haugland, 2002, Molecular Probes, Inc., Eugene, Oregon).

[0105] In some embodiments, the reporter moiety is a chemiluminescent moiety, for example acridinium esters, ruthenium derivatives (e.g., tris(2,2′-bipyridyl) ruthenium), and dioxetanes.

[0106] In some embodiments, the conjugate moiety comprises an affinity or capture tag. Exemplary affinity or capture tag includes, among others, biotin, desthiobiotin, digoxigenin, 3-amino-3-deoxydigoxigenin, and a hapten (e.g., dinitrophenol, Alexa Fluor 40, Alexa Fluor 488, dansyl, Lucifer yellow, Oregon Green 488, fluorescein).

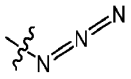
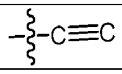
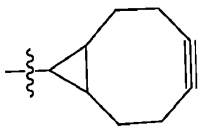
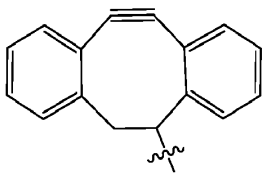
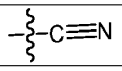
[0107] In some embodiments, the conjugate moiety comprises a peptide. In some embodiments, the peptide comprises a cellular targeting peptide and/or cell penetration peptide (CPP) for enhancing cellular delivery of a conjugate modified oligonucleotide. In some embodiments, the cell penetrating peptide is attached via a linker, including a cleavable linker. Cell penetrating peptides, include among others, TAT, penetratin, MAP, transportan/TP10, VP22, polyarginine, MPG, Pep-1, pVEC, YTA2, YTA4, M918, and CADY. In some embodiments, the conjugate moiety comprises an RGD (Arg-Gly-Asp) peptide. Sequences of some penetrating peptides are described in Copolovici et al., 2014, 8(3):1972–1994 and some are provided below:

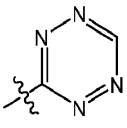
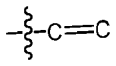
CPP Peptide	Peptide Sequence
TAT	GRKKRRQRRRPPQ
penetratin	RQIKIWFQNRRMKWKK
MAP	KLALKLALKALKAALKLA
Transportan/TP10	GWTLNS/AGYLLGKINLKALAALAKKIL
Transportan9/TP9	LKALLGKIN
VP22	NAKTRRHERRRKLAIER
Polyarginine	(R)n, where n is 8 or 9
MPG	GALFLGFLGAAGSTMGA
Pep-1	KETWWETWWTEWSQPKKKRKV
pVEC	LLIILRRRIRKQAHAHSK
YTA2,	YTAIAWVKAFIRKLRK
YTA4,	IAWVKAFIRKLRKGPLG
M918	MVTVLFRRLRIRRACGPPRVRV
CADY	GLWRALWRLLRSLWRLLWRA
RGD	RGD

[0108] Other cell penetrating peptides, including those conjugated to nucleic acids, are disclosed in, among others, patent publications WO24063570, WO24044663, US2024083949, WO24026141, WO23230600, WO23219933, WO23177261, WO23178327, WO23093960, WO23086342, WO23081893, WO23069332, WO23070108, WO23034515, US2023248630, US2023053924, WO23003380, WO23277628, WO23277575, US2022378946, WO22171972, WO22162200, WO2020144233, WO22180242, WO22132520, WO22129926, WO22125673, WO22120276, WO22101193, US2023287086, US2023357334, US2023144488, and US2023048338; incorporated by reference herein. In some embodiments, the peptide can be attached using a thiol group on the 5'-phosphate of a polynucleotide or oligonucleotide.

[0109] In some aspects, the nucleotide comprising a 3′ blocking moiety further comprises a targeting moiety.

[0110] In some aspects, the nucleotide comprising a 3′ blocking moiety further comprises a reactive moiety. In some aspects, the reactive moiety is on the nucleobase of the nucleotide. In some aspects, the nucleotide comprising a 3′ blocking moiety further comprises a 2′ reactive moiety. In some embodiments, the reactive group is attached to the nucleoside via a linker. In some embodiments, the reactive group is a cyano, azido, alkynyl, amino, carboxyl, sulfhydryl, dibenzocyclooctynyl, vinyl, trans-cyclooctene, or tetrazine. In some embodiments, the reactive group is those used for click chemistry, including copper free click chemistry. Exemplary reactive groups are provided below:

amino	
azido	
alkynyl	
BCN (bicyclo[6.1.0]nonyne)	
dibenzocyclooctynyl	
cyano	

tetrazinyl	
vinyl	

[0111] Other reactive groups used in click chemistry, particularly for nucleic acids, is described in Fantoni et al., Chem. Rev. 2021, 121, 7122–7154, incorporated by reference herein.

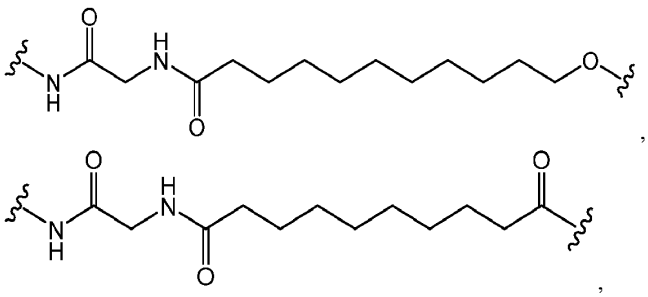
Linker

[0112] In some embodiments, as described above, the conjugate moiety or reactive moiety is attached to the nucleoside or the terminal group through a linker. Various linkers are known in the art for conjugating chemical groups to nucleosides and phosphate groups. Linkers can be, among others, substituted or unsubstituted alkylene, heteroalkylene, alkenylene, heteroalkenylene, arylene, heteroarylene, arylalkylene, arylalkenylene, heteroarylalkylene, heteroarylalkenylene, arylheteroalkylene, arylheteroalkenylene, heteroarylheteroalkylene, and heteroarylalkenylene. In some embodiments, the linker comprises substituted or unsubstituted C_2-C_{22} alkylene, heteroalkylene, or polyethylene glycol. In some embodiments, the linkers have functional groups for conjugation.

[0113] In some embodiments, the linker comprises a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, 1 to 20 carbon atoms, or 1 to 14 carbon atoms, wherein one or more of the carbon atoms in the hydrocarbon chain is optionally replaced by -O-, -NR^1-, -NR^1-C(=O)-, -C(=O)-NR^1, or -S-, and wherein R^1 is hydrogen or (C_1-C_6)alkyl, wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from (C_1-C_6)alkoxy, (C_3-C_6)cycloalkyl, (C_1-C_6)alkanoyl, (C_1-C_6)alkanoyloxy, (C_1-C_6)alkoxycarbonyl, (C_1-C_6)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

[0114] In some embodiments, the L is attached to the nucleoside and/or conjugate through -NH-, -O-, -S-, -(C=O)-, -(C=O)-NH-, -NH-(C=O)-, -(C=O)-O-, -NH-(C=O)-NH-, or -NH-(SO_2)-.

[0115] In some embodiments, the linker L has the structure below:



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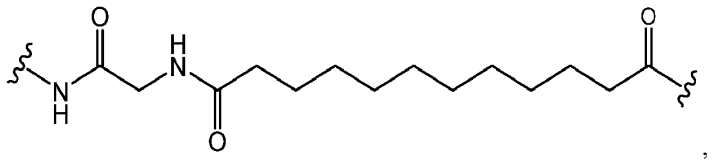
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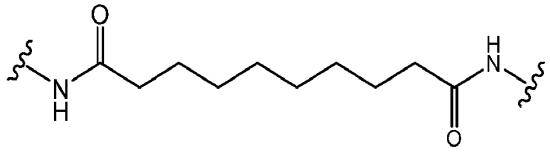
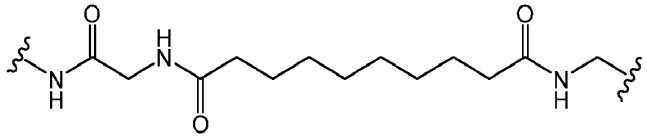
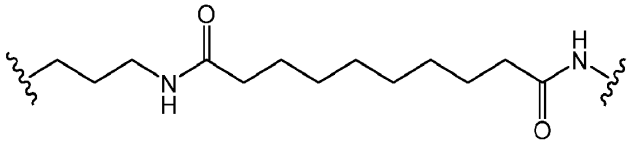
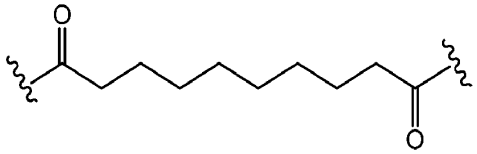
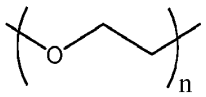

,


, or



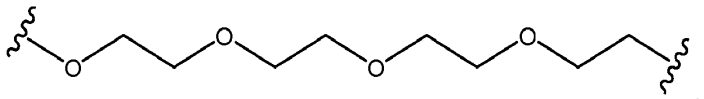


[0116] In some embodiments, the linker comprises a substituted or unsubstituted polyethylene glycol linker. In some embodiments, the polyethylene glycol linker has the formula:



[0117] In some embodiments, n is 2-24. In some embodiments, n is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24.

[0118] In some embodiments, the polyethylene glycol linker has the structure below: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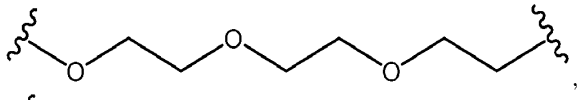
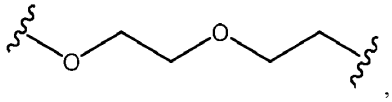
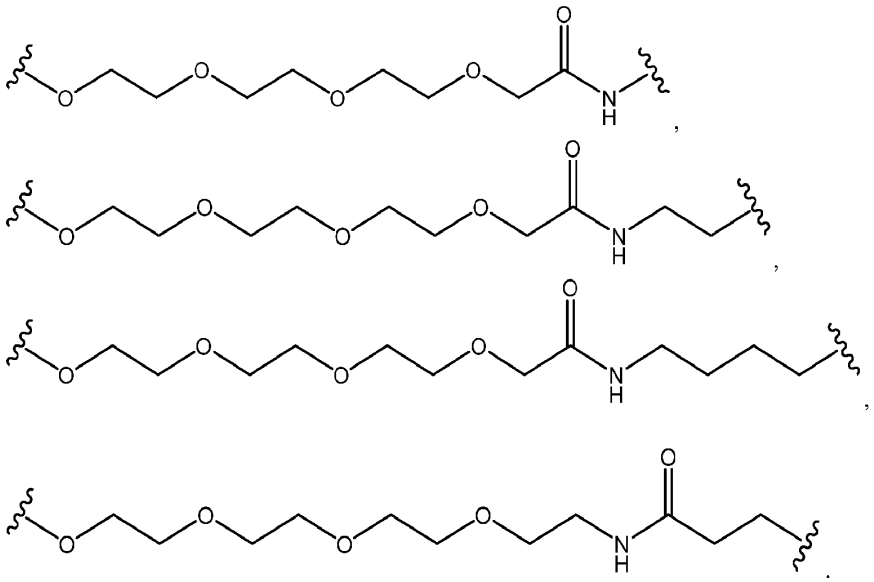

, or


,

[0119] In some embodiments, exemplary polyethylene glycol linkers have the structure below:



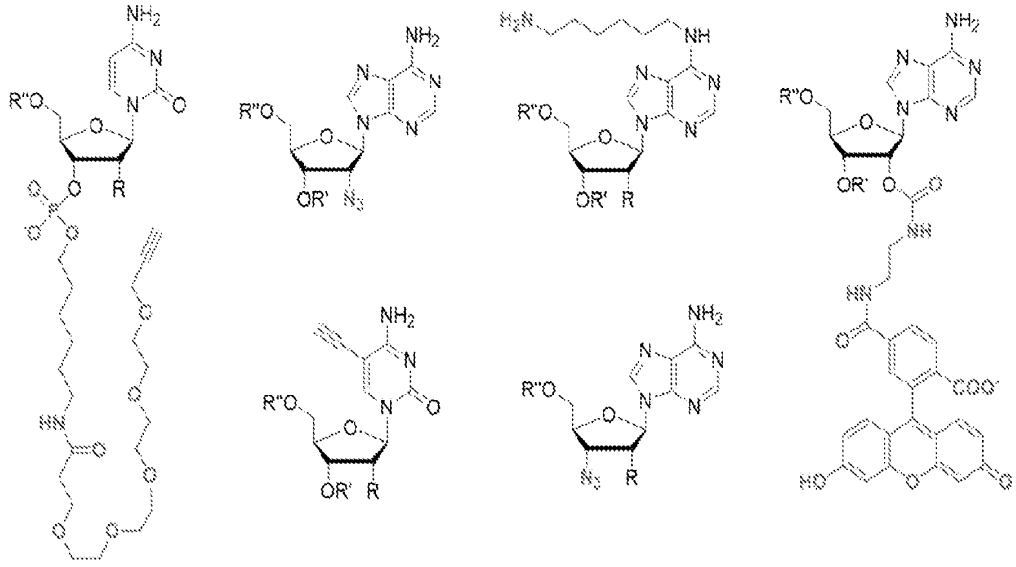
[0120] In some embodiments, the linker is a cleavable linker in which the linker can be cleaved, for example to detach a conjugate moiety. Example of a cleavable linker includes, by way of example and not limitation, a disulfide linkage, enzymatically cleavable linkers (e.g., peptide linkers), and photocleavable linkers (see, e.g., Hermanson, G., Bioconjugate Techniques, 3rd Ed., 2013, Academic Press; see also Bioconjugation Protocols: Strategies and Methods, In Methods in Molecular Biology, 2nd Ed., S.S. Mark ed., 2011, Humana Press).

[0121] In some embodiments, bifunctional linkers can be used to attach a conjugate moiety to the linker and attach the linker-conjugate to the nucleoside or vice versa (see, e.g., Hermanson, G., supra; see also Bioconjugation Protocols: Strategies and Methods, In Methods in Molecular Biology, supra). In some embodiments, an activating group can be attached to an atom to activate the atom to form a covalent bond with another reactive group. Examples of synthetic activating groups that can be attached to an oxygen atom include, but are not limited to, acetate, succinate, triflate, and mesylate. When an activating group is attached to an oxygen atom of a carboxylic acid, the activating group can be a group that is derivable from a known coupling reagent. Examples of such coupling reagents include, but are not limited to, N,N′-dicyclohexylcarbodimide (DCC), hydroxybenzotriazole (HOBt), N-(3-dimethylaminopropyl)-N′-ethylcarbonate (EDC), (denzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU).

Modified nucleotides comprising additional moieties

[0122] In some aspects, the modified nucleotides that may be used by the methods and systems as disclosed herein may comprise targeting moieties, such as targeting moieties that have therapeutic

relevance. In some aspects, the modified nucleotides may comprise reactive and/or sterically bulky side chains. In some aspects, the nucleotides that can be used for the systems and methods as described herein may be modified as shown herein.



Initiating nucleotides acceptors

[0123] The systems and methods provided herein may start from an initiating nucleotide (e.g., an initiating nucleotide acceptor) to generate oligonucleotides that can be elongated in solution (e.g., in a liquid) by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide, for example, by using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety. Depending on the transferase, it may require an initiating oligonucleotide or an initiating nucleotide to act as an acceptor. For example, terminal deoxynucleotidyl transferases (TdTs), poly(N) polymerase, and certain DNA polymerases, such as polymerase □ require initiating oligonucleotides, preferably 4-6 nucleotides in length. Primase, however, is an example of a transferase which is a specialized polymerase that can use nucleotides to generate oligonucleotides that can be further elongated using the systems and methods provided herein.

[0124] In some aspects, the initiating nucleotide is a NTP, NDP, NMP, or a nucleoside. In some aspects, the initiating nucleotide comprises a 5′ blocking moiety. In some aspects, the initiating nucleotide is a NTP, NDP, NMP, or a nucleoside that comprises a 5′ blocking moiety. As a non-limiting example, a 5′ blocking moiety may be used to inhibit primase from using the nucleotide acceptor as the nucleotide donor. In some aspects, the 5′ blocking moiety is a 5′ OH. In some aspects, the initiating nucleotide (e.g., an initiating nucleotide acceptor) is an NTP, which can further serve as a nucleotide donor.

Reaction Chambers

[0125] The systems and methods provided herein comprise reaction chambers. Reaction chambers are vessels in which an enzymatic reaction occurs. More specifically, reaction chambers are vessels or containers. Reaction chambers are made of an inert material and are suited for holding solutions, liquids, and/or reagents during enzymatic reactions or processes. In some aspects, the reaction chamber comprise buffers and/or solutions that may be degassed (e.g., using an vacuum pump) and/or sparged (e.g., an in-line sparger or a reservoir that is operably linked to a sparger or tank configured to flow an inert gas through the buffer and/or solution before or during operation of the system. Exemplary inert gases include argon and nitrogen. In some aspects, the enzymatic reaction or process uses multiple reaction chambers before the enzymatic reaction or process is complete. In some aspects, the enzymatic reaction or process is completed in one reaction chamber. Examples of a reaction or process include, but are not limited to, elongating an oligonucleotide in solution using a transferase and removing the removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide using a hydrolase.

[0126] The reaction chambers contemplated herein enable, promote, maintain, and/or retain favorable conditions for controlled enzymatic reactions or processes (e.g., elongating an oligonucleotide in solution using a transferase, removing the removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety using a hydrolase).

[0127] Reaction chambers contemplated for use include, but not limited to, reaction chambers fabricated from inert or non-reactive plastics, glass, or inert or non-reactive metals. In some aspects, the reaction chambers are coated. In some aspects, the coating comprises one or more polymers. In some aspects, the coating is hydrophilic.

[0128] In some aspects, the reaction chambers are sterile. In some aspects, the reaction chambers are reusable. In some aspects, the reaction chambers are cleanable and/or replaceable. In some aspects, the reaction chambers are single-use.

[0129] The reaction chambers can be of varying sizes and volumes. In some aspects, reaction chambers are suitable for commercial scale production of oligonucleotides. In some aspects, the reaction chambers are 1 mL to 50 mL in volume. In some aspects, the reaction chambers are 50 mL to 250 mL in volume. In some aspects, the reaction chambers are 30 mL to 150 mL in volume. In some aspects, the reaction chambers are 500 mL to 10 L in volume. In some aspects, the reaction chambers are 1 L to 10 L in volume. In some aspects, the reaction chambers are about 5 mL, about 10 mL, about 15 mL, about 50 mL, about 100 mL, about 300 mL, about 500 mL, or about 1 L in volume. In some aspects, the reaction chambers are about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, about 10 L in volume. In some aspects, the reaction chambers are about 1

L, about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, about 10 L in volume.

[0130] In some aspects, the reaction chamber is insulated. In some implementations, the reaction chamber is temperature regulated. The temperature regulation can be achieved by, for example, water recirculation, a thermometer, a heating element, a jacketed reservoir (e.g., a jacketed reagent reservoir such as a jacketed stir tank), a jacketed chamber (e.g., any of the chambers as described herein), or a cooling element. In some aspects, the reaction chamber is maintained at a temperature. In some aspects, the temperature of the reaction chamber is 25 °C, about 30 °C, about 40 °C, about 50 °C, about 60 °C, about 65 °C, about 70 °C, about 75 °C, about 80 °C, about 85 °C, about 90 °C, or about 95 °C. In some aspects, the temperature of the reaction chamber is about 40 °C, about 41 °C, about 42 °C, about 43 °C, about 44 °C, about 45 °C, about 46 °C, about 47 °C, about 48 °C, about 49 °C, or about 50 °C. In some aspects, the temperature of the reaction chamber is 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 aspects, the temperature of the reaction chamber is 40 °C. In some aspects, the first reaction chamber comprising the transferase is about 30°C to about 50 °C (such as about 40 °C). In some aspects, the temperature of the reaction chamber is 50 °C. In some aspects, the temperature of the second reaction chamber (e.g., containing the hydrolase) is about 40 °C to about 60 °C (e.g., about 50 °C).

[0131] In some aspects, the reaction chambers further comprise an apparatus that can be used to stir or mix the solution. In some aspects, the apparatus is an impeller. In some aspects, the apparatus comprises a magnetic stir bar and a stir plate. In some aspects, apparatus is a rod, such as a glass rod.

[0132] In some aspects, the reaction chambers are vessels that can be adapted for flow manufacturing.

[0133] In some aspects, the reaction chambers are batch reaction chambers (any suitable for batch reaction) . Non-limiting examples of the reaction chambers are beakers, drum, conical tubes, microfuge tubes, Erlenmeyer flasks, welled plates, and culture flasks. The reaction chambers can be round bottom, flat bottom, baffled bottom, or conical bottom vessels. Reaction chambers may be cylindrical, spherical, or flask shaped. The vessels typically associated with batch processing can be applied to flow manufacturing methods, for example, by using adapters such as rubber stoppers with tubing. A non-limiting example for using a batch vessel for the systems and processes described herein is adapting an Erlenmeyer flask using a rubber stopper and tubing to form an inlet and an outlet.

[0134] In some aspects, the batch reaction chamber comprises an apparatus for stirring or mixing the solution. Stirring or mixing ensures even distribution of reagents and maintenance of homogeneous conditions throughout the solution. Stirring or mixing is useful for maintaining proper reaction conditions such as by enhancing heat transfer (e.g., preventing heat build up in certain areas of the

reaction chamber), promoting buffer and phase homogeneity and uniform distribution of reactants. These are all factors that influence enzyme reaction kinetics and product yield. In some aspects, the apparatus for stirring or mixing is an impeller. In some aspects, the batch reaction chamber comprises one or more impellers. Impellers are rotating components that can be used to mix the solution in a batch reaction chamber. An impeller is a mechanical agitator and can have different geometries. In some aspects, the impeller is a jet impeller, a blade impeller, a propeller, a paddle or a turbine impeller. In some aspects, the apparatus for stirring or mixing the solution is a rotator or a shaker. A rotator or shaker physically rotates or rocks the batch vessel to create movement in the solution. In some aspects, the apparatus for stirring or mixing the solution is a stir bar and a stir plate. A magnetic stir bar is placed inside of batch reaction chamber and placed on top of a magnetic stir plate, which uses a rotating magnetic field to cause the stir bar to spin or rotate at a set speed.

[0135] Other reaction chambers contemplated herein include rotating bed reactors (such a rotating bed reactor as shown in **FIG. 2A**). Rotating bed reactors are hollow cylinders with a basket inside that is packed with a solid phase or material, such as a packed bed. The basket is immersed in a solution (e.g., a liquid phase or a fluid phase) and spun at a rate to create a flow throughout the basket. Rotating bed reactors are effective for maintaining homogeneous reaction conditions and confer properties such as efficient heat transfer. In some aspects, the packed bed comprises immobilized enzyme on a solid support, such as any of the enzymes described herein. In some aspects, the immobilized enzyme is a transferase or a hydrolase. In some aspects, the immobilized enzyme is a pyrophosphatase. In some aspects, the immobilized enzyme is a transferase, and the transferase is fused to a pyrophosphatase. In some aspects, the packed bed comprises two or more immobilized enzymes. In some aspects, the two or more immobilized enzymes are at least one transferase and at least one pyrophosphatase.

[0136] Other reaction chambers contemplated for use in the systems and methods disclosed herein include columns (such as those shown in **FIG. 2B-2D**). Columns are typically manufactured from a rigid, inert material. Examples include glass columns (e.g., chromatography columns) or columns made of inert plastic. Columns useful for the systems and methods described herein include, but are not limited to, columns from 5-500 cm in length and 0.5 to 10 cm in width. Columns may be capped and operably linked such that they are fluidly connected. The columns may be packed with resin or beads to create a bed. In some aspects, the resin or beads are coupled to enzyme. In some aspects, the enzyme is a transferase. In some aspects, the enzyme is hydrolase. In some aspects, the enzyme is a pyrophosphatase. In some aspects, the enzyme is a transferase fusion, such as a transferase fused to a pyrophosphatase. In some aspects, the enzyme is a transferase-pyrophosphatase fusion. Solutions can migrate through the bed packed in the column via gravity flow or be flowed through using an apparatus that controls flow rate, such as a peristaltic pump.

[0137] In some aspects, the column comprises a bed that can be a fixed bed (e.g., a packed bed) as shown in **FIG. 2B**.

[0138] In some aspects, the bed can be fluidized as shown in **FIG. 2C**. A fluidized bed is when particulates or solids are suspended in solution such that the bed has fluid-like properties.

[0139] In some aspects, the column does not comprise a bed or solid support. In some aspects, the column is used as a vessel to hold the solution. In some aspects, the column comprises free enzyme, such as free transferase (e.g., transferase in solution) or free hydrolase (e.g., hydrolase in solution). In some aspects, the enzyme is retained in the column by a porous medium such as a membrane or a filter. In some aspects, the pores of the membrane or filter comprise pores of a determined diameter. In some aspects, the pores of the membrane or filter have a specific molecular weight cut off (MWCO) value. MWCO permits molecules below a specific size to pass through but retains molecules larger than the cut off size. In some aspects, the column comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the column comprises a membrane that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the column comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide.

[0140] In some aspects, the systems and methods disclosed herein comprise a reaction chamber comprising a transferase, and another reaction chamber comprising a hydrolase. In some aspects, the reaction chambers are batch reaction chambers that optionally include an impeller. In some aspects, one or more of the reaction chambers comprise a rotating bed reactor. In some aspects, the rotating bed reactor is packed with a solid support. In some aspects, the transferase is immobilized on a solid support. In some aspects, the hydrolase is immobilized on a solid support. In some aspects, the reaction chambers are columns, such as fixed bed reactors (see **FIG. 2B**) or fluidized bed reactors (see **FIG. 2C**). In some aspects, the reaction chambers are stirred reactors and comprise at least one impeller (see, for example, **FIG. 2D**). The reaction chambers can be cleanable or replaceable. In some aspects, the reaction chambers comprises an inlet and an outlet.

[0141] In some aspects, the reaction chambers are fluidly connected by one or more conduits. A conduit, such as a conduit that fluidly connects two reaction chambers, may be used to operably link two reaction chambers. In some implementations, the one or more conduits operably links two or more reaction chambers. Non-limiting examples of conduits include chromatography tubing of any material, including glass, plastic, rubber, and silicone. In some aspects, the conduit may be rigid, semi-rigid, or flexible. In some implementations, a conduit may be assembled by connecting two or more conduits. Conduits may be connected using connectors, valves, or fittings. In some aspects, a conduit may be monolithic (e.g., a single, integrated piece). In some aspects, the one or more conduits comprises a set of conduits connecting an outlet of the first reaction chamber to an inlet of the second

reaction chamber. In some aspects, the conduit is capable of bi-directional flow. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber.

[0142] In some aspects, the systems and methods described herein further comprise at least one or more reaction chambers in addition to the first reaction chamber comprising a transferase and the second reaction chamber comprising a hydrolase. Addition reaction chambers (e.g., one or more reaction chambers) comprise enzymes useful for the systems and methods disclosed herein. Additional enzymes include primases, additional transferases, single stranded RNA ligases, or pyrophosphatases. In some aspects, the one or more additional reaction chambers may comprise one or more enzymes. In some aspects, the one or more additional reaction chambers comprise a transferase and a pyrophosphatase.

[0143] In some aspects, the systems and methods described herein further comprise at least a third reaction chamber, at least a fourth reaction chamber, at least a fifth reaction chamber, a least a sixth reaction chamber, at least a seventh reaction chamber. comprising another enzyme, such as a transferase, a single stranded RNA ligase, a primase, or a pyrophosphatase.

[0144] In some aspects, the one or more reaction chamber comprises a second transferase. It is contemplated that some transferase variants will exhibit greater efficiency when conjugating certain nucleotides comprising a 3′ blocking moiety. As such, multiple transferases may be used to conjugate different nucleotides comprising a 3′ blocking moiety, depending on the pre-selected nucleotide. The one or more reaction chambers can be fluidly connected to the other reaction chambers, and the flow path is selected such that the reaction chamber comprising the second transferase is utilized for elongating the oligonucleotide when appropriate.

[0145] In some aspects, the one or more reaction chambers comprises a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. Inorganic phosphate is generated as a byproduct when the transferase conjugates the nucleotide to the oligonucleotide. Inorganic phosphate can inhibit enzymes such as hydrolases used for removing the 3′ blocking moiety. Thus, treating the solution comprising the oligonucleotide to break down inorganic phosphate can be used to maintain efficient reaction kinetics. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the inorganic pyrophosphatase is fused to the transferase. The one or more reaction chambers comprising the pyrophosphatase can be fluidly connected to the other reaction chambers, and the flow path is selected such that the reaction chamber comprising the pyrophosphatase is utilized after the 3′ blocking moiety is removed from the elongated oligonucleotide comprising a 3′ blocking moiety.

[0146] In some aspects, the one or more reaction chamber comprises a primase. Primase is a polymerase that is capable of synthesizing oligonucleotides from single nucleotides or elongating short oligonucleotides. Primase is also more efficient than some hydrolases in elongating oligonucleotides shorter than 10 nucleotides in length. Thus, adding primase in a reaction chamber is useful for synthesizing and/or extending oligonucleotides productive for transferase extension. In some aspects, the one or more reaction chambers comprising the primase can be fluidly connected to the other reaction chambers, and the flow path is selected such that a reaction chamber (e.g., one of the one or more reaction chambers) comprising the primase is utilized to produce an oligonucleotide that can be elongated by the transferase in the first chamber.

[0147] In some aspects, the one or more reaction chamber comprises a single stranded RNA ligase (ssRNA ligase). ssRNA ligase is an enzyme that can ligate a nucleotide donor to an oligonucleotide acceptor, or ligate an oligonucleotide donor to an oligonucleotide acceptor. Elongated nucleotides produced by the systems and methods described herein may be ligated together to produce a longer oligonucleotide. Thus in some aspects, the one or more reaction chambers comprising the ssRNA ligase can be fluidly connected to the other reaction chambers in the system, and the flow path is selected such that a reaction chamber (e.g., one of the one or more reaction chambers) comprising the ssRNA ligase is utilized after an oligonucleotide is elongated by the systems and methods as disclosed herein. In some aspects, one or more oligonucleotides are introduced into the reaction chamber comprising the ssRNA ligase. In some aspects, a nucleotide donor (e.g., a single nucleotide donor or an oligonucleotide donor) is introduced into the reaction chamber comprising the ssRNA ligase along with an oligonucleotide acceptor.

[0148] In some aspects, any of the reaction chambers described herein are fluidly connected by one or more conduits. In some aspects, the first reaction chamber and the second reaction chamber are fluidly connected by one or more conduits. In some aspects, the third reaction chamber is fluidly connected to the first reaction chamber and the second reaction chamber by one or more conduits. A conduit, such as a conduit that fluidly connects two or more reaction chambers, may be used to operably link two or more reaction chambers. In some implementations, the one or more conduits operably links two or more reaction chambers. Non-limiting examples of conduits include chromatography tubing of any material, including glass, plastic, rubber, and silicone. In some aspects, the conduit may be rigid, semi-rigid, or flexible. In some implementations, a conduit may be assembled by connecting two or more conduits. Conduits may be connected using connectors, valves, or fittings. In some aspects, a conduit may be monolithic (e.g., a single, integrated piece). In some aspects, the one or more conduits comprises a set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber. In some aspects, the conduit is capable of bi-directional flow. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a

second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber.

Enzymes

[0149] The enzymes for the process and methods described herein can use wild-type enzymes, engineered enzymes, and combinations of wild-type enzymes and engineered enzymes. Various combinations of such enzymes can be applied to the methods and processes as appropriate where such enzymes are used. In some aspects, any of the enzymes described herein may be modified (e.g., comprise amino acid mutations relative to the wild-type protein) to enhance activity, enhance binding, alter activity (such as, but not limited to activity under different conditions and in the presence of various co-factors) to catalyze a reaction using modified nucleotides, increase product yield, increase protein expression, increase thermoactivity, increase thermostability, increase stability, increase substrate specificity and/or affinity, increase substrate range, increase specific activity, increase resistance to substrate and/or end-product inhibition, increase chemical stability, improve solvent stability, increase solubility, and increase inhibitor resistance or tolerance.

[0150] In some aspects, it is further contemplated that any of the systems or methods using the primase, transferase, single stranded RNA ligase, hydrolase and/or pyrophosphatase can be carried out by retaining the transferase, hydrolase and/or pyrophosphatase in a chamber (e.g., a reaction chamber). Retaining the enzyme can be accomplished through binding or immobilizing the primase, transferase, single stranded RNA ligase, hydrolase and/or pyrophosphatase on a substrate, such as a solid support, a filter (e.g., a porous filter), a porous substrate, a membrane (e.g., a porous membrane), or particles (e.g., beads or resin). Retaining the primase, transferase, single stranded RNA ligase, hydrolase and/or pyrophosphatase permits recycling and reuse of the primase, transferase, single stranded RNA ligase, hydrolase and/or the pyrophosphatase in each iterative cycle until a pre-determined nucleotide sequence is achieved. Retaining the enzyme confers additional cost savings because the enzyme is not used once then discarded. An example of a cycle (e.g., one round of elongating and deblocking the oligonucleotide) is depicted in **FIG. 1**.

[0151] In some aspects, the polypeptide can be entrapped in matrixes or membranes. In some aspects, matrices include polymeric materials such as calcium-alginate, agar, k-carrageenin, polyacrylamide, and collagen. In some aspects, the solid matrices, includes, among others, activated carbon, porous ceramic, and diatomaceous earth. In some aspects, the matrix is a particle, a membrane, or a fiber. Types of membranes include, among others, nylon, cellulose, polysulfone, or polyacrylate.

[0152] In some aspects, the enzymes described herein (such as the enzymes described below) can be fused to a variety of polypeptide sequences, such as, by way of example and not limitation, polypeptide tags that can be used for detection, purification, immobilization on a support medium, or

fusion to another protein. In some aspects, the enzyme/polypeptide comprises an affinity tag. In some aspects, the affinity tag is located at the N-terminus or the C-terminus of the enzyme. In some aspects, the enzyme is fused to a polylysine, for example, for conjugation to a support medium via the amino group of the polylysine. In some aspects, the polylysine is from 2-10 lysine units in length.

[0153] An affinity tag can be used as an adapter to conjugate the enzyme to a solid support (e.g., a resin) that comprises the binding moiety that binds and captures the affinity tag. In some aspects, the affinity tag is a polypeptide tag. Non-limiting examples of a polypeptide tag include a histidine tag (such as a glycine-histidine, penta-histidine or a hexa-histidine tag), a GST tag, a streptavidin tag, a SUMO tag, a MBP tag, a GFP tag, or an epitope tag (such as c-Myc, Flag tag (or iterations thereof), V5, or hemagglutinin (HA)). In some embodiments, the fusion is selected or designed to preserve the activity of the enzyme.

[0154] In some aspects, the affinity tag is recognized by a resin and is used to capture, to purify, to immobilize, and/or to enrich the enzyme by using the tag to immobilize the enzyme to the resin or solid support.

[0155] In some aspects, the enzyme/polypeptide is immobilized on the surface of a support material. In some aspects, the polypeptide is adsorbed on the support material. In some aspects, the enzyme is immobilized on the support material by a covalent, electrostatic, or ionic bond. Support materials include, among others, inorganic materials, such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, and bentonite, or organic materials, such as cellulose (CMC, DEAE-cellulose), starch, activated carbon, polyacrylamide, polystyrene, and ion-exchange resins, such as Amberlite, Sephadex, and Dowex.

[0156] In some aspects, solid supports useful for immobilizing the enzyme/polypeptide in the present disclosure, include beads or resins comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups. Exemplary solid supports useful for immobilizing the enzyme/polypeptide 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).

[0157] Methods of enzyme immobilization are known in the art. The engineered polypeptides can be bound non-covalently or covalently. Various methods for conjugation and immobilization of enzymes to solid supports (e.g., resins, membranes, beads, glass, etc.) are known in the art (see e.g., Yi et al., Proc. Biochem., 2007, 42(5): 895-898; Martin et al., Appl. Microbiol. Biotechnol., 2007, 76(4): 843-85; 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).

Transferase

[0158] A transferase is an enzyme that is capable of elongating an oligonucleotide by covalently ligating a nucleotide or a nucleotide analog (such as a nucleotide comprising a 3′ blocking moiety) to the oligonucleotide. In some aspects, the transferase can react a nucleotide triphosphate (NTP) comprising a 3′ blocking moiety, or an analog thereof comprising a 5′ phosphate analog, with the oligonucleotide to elongate the oligonucleotide. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof is a ribonucleotide. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof is a deoxyribonucleotide. In some aspects, the NTP comprising the 3′ blocking moiety or the analog thereof comprises a 2′ modification. In some aspects, the 2′ modification of the NTP or the analog thereof is 2′-F or 2′-OMe. In some aspects, 3′ blocking moiety of the NTP or the analog thereof is a phosphate moiety.

[0159] In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). TdT is a member of the Pol X family of polymerases. 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. "DNA polymerase mu (Pol μ), homologous to TdT, could act as a DNA mutator in eukaryotic cells" EMBO, 19(7), 1731–1742 (2000).) The TdTs that are contemplate for use in the systems and methods described herein may be engineered (e.g., mutated or comprise amino acid mutations) to 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 TdT or other TdTs or template-independent polymerases known to those of skill in the art. These engineered TdTs are capable of template-independent synthesis of oligonucleotides and polynucleotides. TdTs useful in the systems and methods described herein are disclosed, for example, in PCT/US2023/076667, filed October 12, 2023, which is incorporated by reference herein in its entirety.

[0160] Other template-independent polymerases (e.g., template-independent transferases) (including, but not limited to polyA polymerases, polyU polymerases and terminal urildylytransferases) 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, lambda, polymerase mu, and members of the X family of DNA polymerases) many of which participate in DNA repair processes be used in the systems and methods described herein.

[0161] In some aspects, the transferase comprises a single stranded RNA ligase (ssRNA ligase).

Hydrolase

[0162] A hydrolase is an enzyme that is catalyzes bond cleavage with water. In some aspects, the hydrolase catalyzes the removal of the 3′ blocking moiety from an oligonucleotide comprising a 3′ blocking moiety. Hydrolases within the scope of the invention include, but are not limited to, phosphatases (e.g., alkaline phosphatase) and include, but are not limited to, enzymes acting on ester bonds. Hydrolases contemplated for the methods and systems described herein include phosphatases, such as alkaline phosphatase. Alkaline phosphatase is a dimeric metalloenzyme that hydrolyzes monophosphate esters and generates inorganic phosphate as a byproduct. Alkaline phosphatases are widely distributed enzymes found in both prokaryotes and eukaryotes that catalyze the hydrolysis of phosphate monoesters, with an optimal activity at alkaline pH. In mammals, alkaline phosphatases are present in the intestine (i.e., intestinal alkaline phosphatase) and placenta (i.e., placental alkaline phosphatase). Phosphatases are widely used in molecular biological applications, for example for the removal of 5′ -phosphate from polynucleotides or oligonucleotides for subsequent labeling with labeled ATP, reducing or preventing ligation of polynucleotides or oligonucleotides, and reducing susceptibility of polynucleotides or oligonucleotides to certain nucleases, e.g., l exonuclease. In some aspects, the hydrolase is a recombinant phosphatase. In some aspects, the recombinant phosphatase is used for cleaving phosphate monoesters or analogs thereof. In some aspects, the hydrolase is alkaline phosphatase. In some aspects, the hydrolase is a recombinant alkaline phosphatase. In some aspects, the hydrolase is a recombinant alkaline phosphatase, or a portion thereof. Alkaline phosphatases and variants thereof are disclosed, for example, in U.S. provisional application titled “Recombinant Phosphatases” filed April 16, 2024, and in PCT/US2023/076667, filed October 12, 2023, both of which are incorporated by reference herein in their entirety.

[0163] In some aspects, the hydrolase cleaves a phosphate monoester or analog thereof. In some aspects, the substrate phosphate monoester or analog thereof comprises a nucleotide triphosphate (NTP), a nucleotide diphosphate (NDP), nucleotide monophosphate (NMP), 3’-P-NTP, 3’-P-NDP, 3’-P-NMP, Np, NTP-□-S, NDP-□-S, NMP-□-S, 3’-P-NTP-□-S, 3’-P-NDP-□-S, 3’-P-NMP-□-S, NpS, or any combination thereof. phosphatase and a polynucleotide or oligonucleotide having a 5′-phosphate (5′-P) and/or a 3′-phosphate (3’-P). In some embodiments, the substrate phosphate monoester or analog thereof comprises a polynucleotide or oligonucleotide with a 5′-phosphate, a 3′-phosphate, a 5′-phosphorothioate (5′-S), a 3′-phosphorothioate (3′-S), or any suitable combination

thereof. In some aspects, the hydrolase can remove a 3′ blocking moiety from the oligonucleotide in the solution.

Primase

[0164] In some aspects, the systems and methods disclosed herein comprises the use of a primase, which conjugates a nucleotide acceptor having a 3'-OH group and a nucleotide donor to extend the nucleotide acceptor. In some aspects, the nucleotide acceptor is an initiating nucleotide acceptor as disclosed herein. Primase (e.g., a primase-polymerase) refers to an enzyme in the class of RNA polymerases that is involved in the replication of DNA by catalyzing the synthesis of short RNA molecules from ribonucleoside triphosphates. In some aspects, a primase is a polymerase that can generate oligonucleotides *de novo* from an initiating nucleotide. In some aspects, the primase reacts a nucleotide acceptor having a 3'-OH group with a nucleotide donor. In some aspects, the nucleotide acceptor comprises a polynucleotide acceptor, an oligonucleotide acceptor, or an initiating nucleotide acceptor. In some aspects, the oligonucleotide acceptor is at least 2, 3, 4, 5, 6, 8, 9, 10 nucleotides in length. In some aspects, the polynucleotide or oligonucleotide acceptor is DNA, RNA, or a mixture of DNA and RNA. In some aspects, the 3'-terminal nucleotide of the oligonucleotide acceptor is a ribonucleotide. In some aspects, the 3'-terminal nucleotide of the oligonucleotide acceptor is a deoxyribonucleotide. In some aspects, the initiating nucleotide acceptor comprises NTP, NDP, NMP, or a nucleoside. In some aspects, the nucleotide donor comprises a blocking group or the terminating nucleotide donor comprises a 3'-blocking group to form a 3'-blocked extended polynucleotide or extended oligonucleotide. In some aspects, the primase extends (e.g., elongates) the nucleotide acceptor by attachment of the nucleotide donor to the nucleotide acceptor. some aspects, the primase is a recombinant primase. In some aspects, the recombinant primase comprises a polypeptide fragment of a primase polypeptide, wherein the polypeptide fragment comprises a primase domain. In some aspects, the recombinant primase has template-independent terminal nucleotidyl transferase activity. In some aspects, the recombinant primase has nucleotidyl transferase activity at least for a nucleotide donor dATP, ddATP, ddCTP, ddGTP, 3'-O-methyl ATP, and/or 2'-F-ATP.

[0165] In some aspects, a primase may be used to elongate a nucleotide (e.g., a monomeric nucleotide acceptor) or an oligonucleotide. In some aspects, the oligonucleotide is a 2-mer, a 3-mer, a 4-mer, a 5-mer, a 6-mer, a 7-mer, a 8-mer, a 9-mer, or a 10-mer. In some aspects, the systems and methods disclosed herein comprise a reaction chamber comprising a primase. In some aspects, the primase is a recombinant primase.

[0166] The primase may be a recombinant primase, which may be cloned, produced, and isolated by known methods. The primase need not need a full-length primase, as active polypeptide fragments are suitable for the methods and systems described herein. The primase should include an active primes domain, and moreover should exhibit template-independent terminal nucleotidyl transferase activity.

Primases and variants thereof are disclosed, for example, in U.S. provisional application titled "Recombinant Primases And Methods Of Use" filed April 16, 2024, which is incorporated by reference herein in its entirety.

[0167] In some aspects the primase is retained in a reaction chamber. In some aspects, the primase is immobilized on a solid support. In some aspects, the primase is retained by a filter that prevents passage of the primase and allows passage of a 3′ -blocked donor-acceptor oligonucleotide.

Single stranded RNA ligase (ssRNA ligase)

[0168] Single stranded RNA ligases (ssRNA ligase) can ligate short oligonucleotides to form a longer oligonucleotide in an ATP dependent manner by joining the 5′-PO_4 of a nucleotide donor to the 3′-OH of a nucleotide acceptor. In some aspects, the nucleotide donor is a single nucleotide donor. In some aspects, the nucleotide donor comprises a nucleoside monophosphate. In some aspects, the nucleoside monophosphate is further modified by a 3′-blocking moiety. In some aspects, the nucleotide donor is a pNp. In some aspects, the single nucleotide donor comprises a 5′-phosphate and a 3′-phosphate. In some aspects, the nucleotide donor is an oligonucleotide donor. In some aspects, the oligonucleotide donor comprises a 5′-phosphate and a 3′-phosphate. In some aspects, the nucleotide acceptor is an oligonucleotide. In some aspects, the oligonucleotide comprises a 5′-OH. In some aspects, the oligonucleotide comprises a 5′ blocking moiety.

[0169] In some aspects, the systems and methods disclosed herein comprise a reaction chamber comprising a ssRNA ligase, such as those disclosed in U.S. provisional application titled "Methods of RNA ligase mediated oligonucleotide synthesis," filed April 16, 2024, which is incorporated by reference herein in its entirety.

[0170] In some aspects the ssRNA ligase is retained in a reaction chamber. In some aspects, the ssRNA ligase is immobilized on a solid support. In some aspects, the ssRNA ligase is retained by a filter that prevents passage of the primase and allows passage of a 3′ -blocked donor-acceptor oligonucleotide.

Pyrophosphatase

[0171] In some aspects, the present disclosure further provides auxiliary enzymes for template-free synthesis of an oligonucleotide. One such auxiliary enzyme is pyrophosphatase such as inorganic pyrophosphatase, which catalyzes the conversion of conversion of inorganic pyrophosphate (PPi) to two orthophosphate ions (Pi). In some aspects, the systems and methods disclosed herein provide for the use of IPP enzymes (e.g., recombinant IPP enzymes) with higher specific activity, reduced off-target hydrolysis and other side reactions, increased thermostability, and increased solubility compared to type I IPP enzymes.

[0172] Pyrophosphate is often released from reactions that utilize nucleoside triphosphates (NTP). Many cellular enzymes are capable of catalyzing pyrophosphate conversion; however, the activity of most of these enzymes is not specific to a pyrophosphate substrate. For example, alkaline phosphatase will fully degrade pyrophosphate but will also hydrolyze other phosphates including the 5′-phosphates of nucleotides. Inorganic pyrophosphatases (IPP enzymes or IPPases) are known to have both high activity in the conversion of pyrophosphate to orthophosphate and relatively low activity on other phosphorylated substrates including, but not limited to, NTPs and sugar phosphates. Inorganic pyrophosphate is also a known inhibitor of hydrolases such as alkaline phosphatase, and accumulation can block alkaline phosphatase activity, which interferes with the hydrolase-mediated removal of the 3′ blocking moiety from the NTP. In some aspects, the systems and methods disclosed herein comprise a reaction chamber comprising an inorganic pyrophosphatase. There are three types of inorganic pyrophosphatases (Type I, Type II, Type III). Type I IPPs have been used commercially in coupled reactions to facilitate enzymatic reactions involving DNA/RNA polymerase and other reactions that produce pyrophosphate. Type I IPP enzymes generally drive the hydrolysis of pyrophosphate to release heat, the H(+)-PPases couple the energy of inorganic pyrophosphate hydrolysis to proton movement across biological membranes. However, these enzymes are not readily conducive to *in vitro* and industrial processes. Type II IPP enzymes have generally been characterized from bacterial and archaeal sources (see for example, Zyryanov et al. "Rates of Elementary Catalytic Steps for Different Metal Forms of the Family II Pyrophosphatase from Streptococcus gordonii." Biochemistry 43(4):1065-1074 (2004), Gajadeera et al. "Structure of inorganic pyrophosphatase from Staphylococcus aureus reveals conformational flexibility of the active site." J Struct Biol. 189(2):81-86 (2015), Parfenyev et al. "Quaternary Structure and Metal Ion Requirement of Family II Pyrophosphatases from Bacillus subtilis, Streptococcus gordonii, and Streptococcus mutans*" J Biol Chem. ;276(27):24511-24518 (2001)). It has been suggested that Type II IPP enzymes may prefer manganese and/or cobalt in their active sites (Zyryanov et al. "Mechanism by which metal cofactors control substrate specificity in pyrophosphatase." Biochemical Journal. 367(Pt 3):901-906 (2002)), may have higher specific activity than type I IPP enzymes, and may be more selective toward pyrophosphate and have lower activity toward other substrates, such as NTPs, compared to type I IPP enzymes (Baykov et al. "Inorganic pyrophosphatases of Family II—two decades after their discovery." FEBS Letters 591(20):3225-3234 (2017)). Thus in some aspects, the systems and methods described herein include the use of Type II IPPs or Type I and Type II IPP variants thereof (such as those disclosed in U.S. provisional application titled "Uses of Type II Inorganic Pyrophosphatases, filed April 16, 2024, incorporated by reference herein in its entirety).

[0173] In some aspects, any of the enzymes described herein can be provided as a fusion protein. In some aspects, the inorganic pyrophosphatase is provided as a fusion to a transferase. In some aspects, the enzyme is fused to a variety of polypeptide sequences, such as, by way of example and not

limitation, polypeptide tags that can be used for detection and/or purification. In some aspects, the fusion polypeptide of the enzyme comprises a glycine-histidine or histidine-tag (His-tag). In some aspects, the fusion polypeptide of the enzyme comprises an epitope tag, such as c-myc, FLAG, V5, or hemagglutinin (HA). In some aspects, the fusion polypeptide of the enzyme comprises a GST, SUMO, Strep, MBP, or GFP tag. In some aspects, the fusion is to the amino (N-) terminus of engineered enzyme polypeptide. In some aspects, the fusion is to the carboxy (C-) terminus of the enzyme polypeptide.

[0174] In some aspects, the fusion polypeptide comprises a transferase and the pyrophosphatase. In some aspects, the pyrophosphatase is fused to the C terminus of the transferase. In some aspects, the transferase and the pyrophosphatase are connected by a polypeptide linker.

Purification Chambers

[0175] In some aspects, the systems and methods described herein further comprise one or more purification chambers. In some aspects, the systems and methods described herein may comprise two or more different types of purification chambers, such as two or more of the types of purification chambers as described herein. Purification chambers can be used to purify the oligonucleotide from reaction byproducts such as inorganic phosphate, or remove unreacted nucleotides comprising a 3′ blocking moiety from the solution. In some aspects, the purification chambers isolate and/or substantially separate the oligonucleotide from other reagents and reaction byproducts. In some aspects, a purification chamber can be used to substantially separate and/or isolate at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the total mole or percent amount of oligonucleotide from other reagents (e.g., enzymes) and reaction byproducts after the reservoirs. Purification chambers can be vessels or chambers, and are composed of an inert material. In some aspects, the purification chambers are columns. In some aspects, purification chamber comprises a column. In some aspects, the purification chamber comprises a liquid chromatography column. Liquid chromatography comprises a mobile phase and a stationary phase, and certain molecules are retained in the stationary phase based on their inherent properties while others prefer to travel with the mobile phase. Based on these properties, molecules will exit the chromatography column at different times or in different fractions. In some aspects, the purification chamber comprises a size exclusion chromatography. Size exclusion chromatography separates molecules by parameters such as molecular weight and hydrodynamic radius. In some aspects, the purification chamber comprises an ion exchange column. Ion exchange separates molecules by inherent charge parameters when the target molecule (e.g., the oligonucleotide) is in different buffers that modify whether the molecule prefers to interact more or less with the stationary phase. In some aspects, the purification chamber is a reverse phase column. Reverse phase chromatography relies on polar/nonpolar characteristics of the molecule for separation of the target molecule from the mixture. In some aspects, the purification

chamber is part of a tangential flow filtration system. A tangential flow filtration system involves using a filter to remove or separate contaminating particles from samples.

[0176] The purification chamber may be configured to remove salts, reaction byproducts and/or unreacted reagents, for example as a desalting chamber. Desalting chambers are useful for separating the oligonucleotide from buffer components such as salts, small molecules (such as small molecule byproducts), impurities and/or metals. Desalting chambers are also useful for buffer exchanging the oligonucleotide, and for concentrating the oligonucleotide into a smaller volume. Desalting chambers may be used outside of the flow system, for example by collecting the product and desalting it prior to re-introducing the oligonucleotide for further reactions or collecting the product at the end of oligonucleotide synthesis. Desalting chambers that are not coupled to the systems as described herein include spin cartridge filters. The sample is applied to the spin cartridge filter, and centrifugal force is applied to the unit to draw the permeate through the filter. The filter comprises pores of a specified molecular weight cut off, such that any of the components of the sample that are larger than the size of the cut off are significantly more retained in the retentate, and components of the sample that are smaller than the size of the cut off are flowed through with the permeate. Desalting chambers may also be used as a part of the flow system, such as a tangential flow system or a cross flow filtration system. Filtration techniques, such as ultrafiltration, may be used, and the cartridges or conditions (for example, the flow rate to maintain appropriate transmembrane pressure, or the membrane surface required for appropriate permeate flux) are compound (e.g., product) dependent. The oligonucleotide (such as an oligonucleotide of about 9 or about 10 nucleotides in length or more) is retained with the retentate, but the permeate is flowed to the waste or discarded. In some aspects, the oligonucleotide is desalted using a desalting chamber as needed. In some aspects, the oligonucleotide is desalted at defined intervals and/or at defined cycle numbers. is In some aspects, the desalting chamber is a vessel, e.g., a column that is packed with resin for desalting and buffer exchange, such as G25 resin. In some aspects, the desalting chamber is a cartridge with a membrane of a designated molecular weight cut off. In some aspects, desalting chamber is a tangential flow device or a ultrafiltration and/or diafiltration device (e.g., cassette), such as those used in ultrafiltration/diafiltration (UF/DF). Use of ultrafiltration/ diafiltration methods (e.g., use of ultrafiltration, diafiltration cassettes) also concentrates the oligonucleotides while substantially separating it from salts, reaction byproducts and/or unreacted reagents. In some aspects, the cassette comprises pores with a molecular weight cut off of 1 kilodaltons. In some aspects, the cassette comprises pores with a molecular weight cut off 3 kilodaltons. In some aspects, the desalting chamber comprises a membrane. In some aspects, the membrane is a polyethersulfone (PES) membrane. In some aspects, the temperature of the desalting chamber is 25 °C. In some aspects, the desalting chamber is cleanable. In some aspects, the desalting chamber is cleaned in place. In some aspects, the desalting chamber is reusable. In some aspects, the desalting chamber is replaceable. In some aspects, the desalting chamber is disposable.

Enzyme capture chambers

[0177] In some aspects, the systems and methods described herein further comprise one or more enzyme capture chambers. Enzyme capture chambers can be used to further separate trace levels of enzymes that were not retained from the reaction reservoirs. Trace amounts of the enzymes disclosed herein may not be retained by the reaction chambers. For example, the transferase or the hydrolase may be immobilized to a solid support, but the enzyme or its active subunits may decouple during the cycle run. Thus, the enzymes described herein may be further modified with an affinity tag or another purification tag to capture any free enzyme in solution. A chamber, such as an in-line column, may be placed at the outlet of the reaction chamber (e.g., downstream of the reaction chamber) to bind any enzyme that is flowed in solution with the oligonucleotide. In some aspects, the chamber comprises a solid support that captures the enzyme and allows passage of the oligonucleotide. The resin may be, for example, an ion exchange resin or an affinity resin that can capture the enzyme. For example, the enzyme may include a His-tag and the resin may be nickel, cobalt, or IMAC resin. In some aspects, the enzyme capture chambers to further retain, separate, or exclude the enzyme from the solution comprising the oligonucleotide. In some aspects, the enzyme capture chambers further remove, separate and/or isolate the enzyme such that at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% of the enzyme is retained or removed from the solution comprising the oligonucleotide.

Reservoirs

[0178] In some aspects, the systems and methods described herein further comprise one or more reagent reservoirs. Reagent reservoirs are used to hold solutions, buffers, or mixtures that provide essential reagents for template-free oligonucleotide synthesis. Reagents include co-factors, buffers, and nucleotides comprising 3′ blocking moiety. In some aspects, the reagent reservoir comprises an impeller, such as an impeller as described herein. In some aspects, the reagent reservoir is replaceable or cleanable. The reagent reservoir may comprise an outlet. In some aspects, the reagent reservoir comprises an inlet and an outlet. In some aspects the reagent reservoir is fluidly connected to any of the reaction chambers or product reservoirs as described herein. In some aspects, the reagent reservoir is insulated. In some aspects, the reagent reservoir is temperature regulated. The temperature regulation can be achieved by, for example, a jacketed reservoir (e.g., a jacketed reagent reservoir such as a jacketed stir tank), or a cooling element. In some aspects, the reagent reservoir is maintained at a temperature. In some aspects, the temperature of the reagent reservoir is about 2 °C to 8 °C. In some aspects, the temperature of the reagent reservoir is about 2 °C, about 3 °C, about 4 °C, about 5 °C, about 6 °C, about 7 °C, or about 8 °C. In some aspects, the temperature of the reagent reservoir is 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C. In some aspects, the temperature of the reagent reservoir is 2 °C. In some aspects, the temperature of the reagent reservoir is 3 °C. In some aspects, the temperature of the reagent reservoir is 4 °C. In some aspects, the temperature of the reagent reservoir

is 5 °C. In some aspects, the temperature of the reagent reservoir is 6 °C. In some aspects, the temperature of the reagent reservoir is 7 °C. In some aspects, the temperature of the reagent reservoir is 8 °C.

[0179] In some aspects, the systems and methods described herein further comprise one or more product reservoirs. In some aspects, the product reservoir holds the product and any byproducts that are produced from the enzymatic reaction. In some aspects, the product reservoir comprises an inlet. In some aspects, the product reservoir comprising an inlet and an outlet. In some aspects, the system comprises a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the reagent reservoir. In some aspects, the product reservoir comprises an impeller. In some aspects, the product reservoir is replaceable or cleanable. In some aspects, the product reservoir is insulated. In some aspects, the product reservoir is temperature regulated. The temperature regulation can be achieved by, for example, a jacketed reservoir (e.g., a jacketed product reservoir such as a jacketed stir tank), or a cooling element. In some aspects, the product reservoir is maintained at a temperature. In some aspects, the temperature of the product reservoir is about 2 °C to 8 °C. In some aspects, the temperature of the product reservoir is about 2 °C, about 3 °C, about 4 °C, about 5 °C, about 6 °C, about 7 °C, or about 8 °C. In some aspects, the temperature of the product reservoir is 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C. In some aspects, the temperature of the product reservoir is 2 °C. In some aspects, the temperature of the product reservoir is 3 °C. In some aspects, the temperature of the product reservoir is 4 °C. In some aspects, the temperature of the product reservoir is 5 °C. In some aspects, the temperature of the product reservoir is 6 °C. In some aspects, the temperature of the product reservoir is 7 °C. In some aspects, the temperature of the product reservoir is 8 °C.

Systems for Template-Free Synthesis of an Oligonucleotide

[0180] The present disclosure describes systems for template-free synthesis of an oligonucleotide. Also described herein are systems for performing the methods described herein. In some aspects, the system comprises a first reaction chamber and a second reaction chamber. In some aspects, the system includes one or more reaction chambers. In some aspects, the system comprises two or more reaction chambers. In some aspects, the system comprises three or more reaction chambers. In some aspects, the system comprises four or more reaction chambers. In some aspects, the system further comprises any of the reagent reservoirs, product reservoirs, and purification chambers as described herein. In some aspects, the systems described herein includes mechanisms for limiting oxidation, such as by limiting or removing oxygen from the system. Mechanisms for preventing oxidation include the use of inert gasses (such as argon or nitrogen) for inerting, purging, or sparging. In some aspects, the systems described herein may be operated in an inert atmosphere or under inert atmospheric conditions.

[0181] In some aspects, provided herein is system for template-free synthesis of an oligonucleotide comprising: a first reaction chamber comprising a transferase; and a second reaction chamber comprising a hydrolase; wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber. In some aspects, the first reaction chamber comprises a transferase. In some aspects, the transferase is a TdT. In some aspects, the transferase is a member of the Pol X family. In some aspects, the first reaction chamber further comprises a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the first reaction chamber comprises a transferase-pyrophosphatase fusion. In some aspects, the second reaction chamber comprises a hydrolase. In some aspects, the hydrolase is a phosphatase. In some aspects, the phosphatase is an alkaline phosphatase.

[0182] In some aspects, the systems described herein may be operated in parallel. For example, multiple and identical reaction chambers may be operated in parallel (e.g., simultaneously operated). In some aspects, the systems described herein may be operated serially.

[0183] In some aspects, the first reaction chamber or the second reaction chamber is a column. In some aspects, the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support. In some aspects, the first reaction chamber comprises a fluidized bed comprising the transferase immobilized on a solid support. In some aspects, the fluidized bed further comprises a pyrophosphatase, such as an inorganic pyrophosphatase. In some aspects, the first reaction chamber comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide. In some aspects, the first reaction chamber comprises a filter that prevents passage of the transferase and the pyrophosphatase and allows passage of the oligonucleotide. In some aspects, the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a fluidized bed comprising the hydrolase immobilized on a solid support. In some aspects, the second reaction chamber comprises a filter that prevents passage of the hydrolase and allows passage of the oligonucleotide.

[0184] In some aspects, the first reaction chamber or the second reaction chamber is a batch reaction chamber. In some aspects, the batch reaction chamber comprises an impeller. In some aspects, the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support. In some aspects, wherein the second reaction chamber comprises a rotating bed reactor comprising the hydrolase immobilized on a solid support.

[0185] The system can further comprise a temperature regulator. The use of varied temperatures may be beneficial, for example, to optimize enzyme activity or promote substrate/product stability. Temperature can also impact liquid viscosity, product separation, and product polishing. These

parameters impact the efficiency and yield of each cycle of determined nucleotide addition to reach an oligonucleotide of a pre-defined sequence. Temperature regulators can be column wall thermostats, a still air thermostat, forced air thermostats, passive pre-heaters, active pre-heaters, post-column liquid phase cooler, or heat exchangers. In some aspects, the temperature regulators can also be thermal blankets or insulation sleeves, jacketed reservoirs or chambers (such as any of the reaction chambers, purification chambers, enzyme capture chambers, and/or a reagent reservoirs as described herein), water baths or ice baths. Each of these elements may also be coupled to thermometers to monitor heat or cooling. In some aspects, the temperature regulator is configured to control the temperature of the solution in the first reaction chamber or the second reaction chamber. In some aspects, the system comprises an in-line temperature regulator that controls the temperature of the solution in at least one of the one or more conduits. Thus in some aspects, the system further comprises a temperature regulator that controls the temperature of the solution in the system. In some aspects, the temperature regulator is configured to control the temperature of the solution in the first reaction chamber or the second reaction chamber. In some aspects, the temperature regulator is a jacketed chamber, such as a jacketed purification chamber. In some aspects, the temperature regulator is a jacketed reaction chamber. In some aspects, the temperature regulator is a jacketed reagent reservoir. In some aspects, the temperature regulator is a jacketed stir tank.

[0186] Mechanisms of degassing the buffers or solutions may also be incorporated into the systems as described herein. In some aspects, the reagent reservoirs may be operably linked to an inert gas source, such as nitrogen or argon gas tanks to sparge the buffer and/or reagents prior to introduction into the system. Sparging with inert gasses removes oxygen from the buffers, reagents, and/or liquids in the system. In-line degassing apparatuses, such as an in-line vacuum pump) may be optionally included in the systems as disclosed herein. Degassing the buffers and/or solutions used in the system may be useful, for example, for preventing oxidation of oligonucleotides and/or the modified nucleotides used to make oligonucleotides. In some aspects, the systems optionally include one or more in-line degassers for degassing the buffers and/or solutions. In some aspects, the system optionally includes one or more reagent reservoirs that are operably linked to a tank comprising an inert gas (such as nitrogen or argon) such that the contents within the reservoir are sparged prior to introduction into the systems. In some aspects, the system may include one or more in-line spargers for sparging the fluids within the system. In some aspects, the buffers and/or solutions comprise reagents, such as reagents that are useful for template-independent oligonucleotide synthesis.

[0187] In some aspects, the system comprises one or more conduits that connects the first reaction chamber and the second reaction chamber. In some aspects, the conduits comprise an in-line temperature regulator that controls a temperature of the solution in at least one of the one or more conduits. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of

conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber. In some implementations, the system for template free synthesis of an oligonucleotide comprises a first reaction chamber, and a second reaction chamber. In some implementations, the system is configured to flow the oligonucleotide in a liquid phase from the first reaction chamber to the second reaction chamber. In some implementations, the system is configured to flow the oligonucleotide in the liquid phase from the second reaction chamber back to the first reaction chamber.

[0188] **FIG. 3** illustrates an example of a system in accordance with one implementation, wherein the system comprises a first reaction chamber comprising a transferase 300 and a second reaction chamber 302 comprising a hydrolase. The system can be configured such that the reservoirs can be configured to flow an oligonucleotide in solution from the first reaction chamber 300 to the second reaction chamber 302 while retaining the transferase in the first reaction chamber 300, and the oligonucleotide can be flowed from the second reaction chamber 302 to the first reaction chamber 300 while retaining the hydrolase in the second reaction chamber 302. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. In this exemplary system, the reaction chambers are batch vessels that are fluidly connected by conduit 304.

[0189] In this system, the oligonucleotide is introduced to the system by addition to first reaction chamber 300. In some implementations, the reservoir comprises an apparatus for mixing solutions in first reaction chamber 300. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber 300. The solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety is then flowed through conduit 304 into second reaction chamber 302. The first reaction chamber 300 comprises a transferase which conjugates the nucleotide comprising a 3′ blocking moiety (such as, for example, an NQP) to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduit 304. In some implementations, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduit 304, and into second reaction chamber 302 comprises a hydrolase and the transferase is retained in the first reaction chamber 300. Different configurations of the system can be designed to retain the transferase, such as by a membrane positioned near the outlet of first reaction chamber 300. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 300. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of first reaction chamber 300. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 302 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated

oligonucleotide is then flowed through conduits 304 to first reaction chamber 300. In some implementations, the elongated oligonucleotide is flowed to first reaction chamber 300 and the hydrolase is retained in second reaction chamber 302. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 302. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 302. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 302. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to first reaction chamber 300 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) The system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into first reaction chamber 300, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may also comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 300 or second reaction chamber 302) in the system (e.g., into first reaction chamber 300 or second reaction chamber 302). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange

and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 300 and the second reaction chamber comprising the hydrolase 302). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 300 and the second reaction chamber comprising the hydrolase 302). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-

limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system can further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0190] The system may further comprise one or more valves. Valves are used for multiple purposes in a liquid delivery system. Non-limiting examples of valves that can be used in the system include injection or inlet valves that are used to introduce liquid sample into the system, flow path switching valves that may be used to change the flow path of the liquid, column valves for attachment of one or more columns to the system, and outlet valves for waste or sample collection. In some aspects, the one or more valves are multiposition rotary valves. In some aspects, the valves are single position valves. In some aspects, the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system. In some aspects, the system comprises one or more diverter valves. In some aspects, the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system. In some aspects, the systems disclosed herein comprise diverter valves configured to alternatively direct flow of the solution through (i) a first flow pathways comprising flow of the solution from the outlet of the first reaction chamber to an inlet of the purification chamber, and from an outlet of the purification chamber to the inlet of the first reaction chamber, or (ii) a second flow pathway comprising flow of the solution from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from an outlet of the second reaction chamber to the inlet of the first reaction chamber.

[0191] **FIG. 4** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 400 and a second reaction chamber 402 comprising a hydrolase. The system can be configured such that the reservoirs can be configured to flow an oligonucleotide in solution from the first reaction chamber 400 to the second reaction chamber 402 while retaining the transferase in the first reaction chamber 400, and the oligonucleotide can be flowed from the second reaction chamber 402 to the first reaction chamber 400 while retaining the hydrolase in the second reaction chamber 402. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. In the system, the

reaction chambers are vessels such as batch vessels that are fluidly connected by conduit 404, but the flow is modulated by control valve 406 .

[0192] **FIG. 4** illustrates an example of a system in accordance with one implementation. The oligonucleotide is introduced to the system by addition to a first reaction chamber 400. In this particular embodiment, the solution is flowed from the first reaction chamber 400 to the second reaction chamber 402 via conduit 404. Flow is applied by gravity, and controlled by control valve 406. In some embodiments, the transferase is added to first reaction chamber 400. After oligonucleotide elongation, the oligonucleotide comprising the 3′ blocking moiety is flowed to the second chamber through control valve 406 and conduit 404. The transferase is retained in the top chamber, optionally through immobilization, a membrane or a filter. The hydrolase is added to second reaction chamber 402 and the elongated oligonucleotide is deblocked. Product can be separated from the hydrolase by, for example, inverting the chambers and using gravity flow to re-cycle the solution into the first reaction chamber 400. In some aspects, the hydrolase is retained in the second reaction chamber by gravity flow through a filter or a membrane, immobilized enzyme, or any combination thereof.

[0193] The system may further comprise one or more pumps configured to flow the solution or the liquid phase. A pump is a solvent delivery system. A pump can deliver a single solvent or multiple solvents. In some implementations, the pump comprises a mixer that combines multiple solvents into a final solvent that can be delivered to the system. Pumps may be configured to deliver the solvents at a determined flow rate. In some aspects, the one or more pumps deliver solvent at a constant flow rate. In some implementations, the one or more pumps are configured to control a flow rate of the liquid phase. In some implementations, the system further comprises one or more pumps configured to flow the liquid phase from the first reaction chamber to the second reaction chamber, and from the second reaction chamber to the first reaction chamber. In some aspects, the system further comprises one or more pumps configured to flow the solution from the first reaction chamber to the second reaction chamber, and from the second reaction chamber to the first reaction chamber. In some aspects, the one or more pumps are configured to control a flow rate of the solution. In some aspects, the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system.

[0194] **FIG. 5** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 500 and a second reaction chamber 502 comprising a hydrolase. The system can be configured such that the reservoirs can be configured to flow an oligonucleotide in solution from the first reaction chamber 500 to the second reaction chamber 502 while retaining the transferase in the first reaction chamber 500 and the oligonucleotide can be flowed from the second reaction chamber 502 to the first reaction chamber 500 while retaining the hydrolase in the second reaction chamber. Reservoirs and reaction

chambers can be, for example, batch vessels, flasks, or in line columns. In the system, the reaction chambers are batch vessels that are fluidly connected by conduits 504 and 504, and the flow is controlled by pump 508.

[0195] In the system of **FIG. 5**, the oligonucleotide is introduced to the system by addition to first reaction chamber 500. In some implementations, the reservoir comprises an apparatus for mixing solutions in first reaction chamber 500. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber comprising a transferase 500. The solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety is then flowed through conduit 504 into second reaction chamber comprising a hydrolase 502. The movement of the solution, direction of the flow, and flow rate are parameters that can be controlled by optional components such as valves and pumps, such as pump 506. The first reaction chamber 500 comprises a transferase which conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduit 504. In some implementations, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduit 504, and into second reaction chamber 502 and the transferase is retained in the first reaction chamber 500. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 500. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 500. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 500. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 402 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated oligonucleotide is then flowed through conduits 504 to first reaction chamber 500. In some implementations, the elongated oligonucleotide is flowed to first reaction chamber 500 and the hydrolase is retained in second reaction chamber 502. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 502. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 502. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 502. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to first reaction

chamber 500 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP). The system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into first reaction chamber 500, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 500 or second reaction chamber 502) in the system (e.g., into first reaction chamber 500 or second reaction chamber 502). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 500 and the second reaction chamber comprising the hydrolase 502). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system

comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 500 and the second reaction chamber comprising the hydrolase 502). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the

interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0196] **FIG. 6** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 600 and a second reaction chamber 602 comprising a hydrolase. The system can be configured such that the reservoirs can be configured to flow an oligonucleotide in solution from the first reaction chamber 600 to the second reaction chamber 602 while retaining the transferase in the first reaction chamber 600, and the oligonucleotide can be flowed from the second reaction chamber 602 to the first reaction chamber 600 while retaining the hydrolase in the second reaction chamber 602 . Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. In the system, the reaction chambers are batch vessels that are fluidly connected by conduits 604 and 606, and the direction of the flow is controlled by control valve 608. The reaction chambers are also fluidly connected by conduits 618 and 620, and the direction of the flow is controlled by control valve 616. In some aspects, the flow can be unidirectional through each of these conduits such that the flow path for flowing the solution from first reaction chamber 600 to second reaction chamber 602 is not the same flow path for flowing the solution from second reaction chamber 602 to first reaction chamber 600. In some aspects, the pathways can be modified using control valves 616 and 608 such that the solution can cycle through a reaction chamber (e.g., the first reaction container 600 or second reaction container 602) before advancing to the other reaction chamber.

[0197] In the system of **FIG. 5**, the oligonucleotide is introduced to the system by addition to first reaction chamber 600. In some implementations, the reservoir comprises an apparatus for mixing solutions in first reaction chamber 600. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber comprising a transferase 600. The solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety is then flowed through conduit 604 into second reaction chamber comprising a hydrolase 602. The movement of the solution, direction of the flow, and flow rate are parameters that can be controlled by optional components such as valves and pumps, such as pump 610. The first reaction chamber 600 comprises a transferase which conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduit 604 and 606. In some implementations, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduit 604 and 606 through control valve 608, and into second reaction chamber 602 and the transferase is retained in the first reaction chamber 600. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 600. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 600. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations,

the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 600. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 602 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated oligonucleotide is then flowed through conduits 604 to first reaction chamber 600. In some implementations, the elongated oligonucleotide is flowed to first reaction chamber 600 and the hydrolase is retained in second reaction chamber 602. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 602. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 602. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 602. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to first reaction chamber 600 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) The system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into first reaction chamber 600, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 600 or second reaction chamber 602) in the system (e.g., into first reaction chamber 600 or second reaction chamber 602). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one

or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 600 and the second reaction chamber comprising the hydrolase 602). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 600 and the second reaction chamber comprising the hydrolase 602). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more

analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0198] In some aspects, the system further comprises a third reaction chamber comprising an enzyme. In some aspects, the system comprises a third reaction chamber that comprises a transferase. In some aspects, the system comprises a third reaction chamber that comprises a second transferase. In some aspects, the system further comprises a third reaction chamber comprising a second transferase, wherein the system is further configured to selectively flow said oligonucleotide in the solution from the second chamber to the third reaction chamber while retaining the hydrolase in the second reaction chamber, and flow the oligonucleotide in the solution from the third reaction chamber to the second reaction chamber while retaining the second transferase in the third reaction chamber. In some aspects, the transferase and the second transferase are different types of transferase. In some aspects, wherein the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system. In some aspects, the third reaction chamber comprises a pyrophosphatase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the pyrophosphatase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the pyrophosphatase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the pyrophosphate is retained in the third reaction chamber comprising the pyrophosphatase.

[0199] Thus in some aspects, provided herein is a system for template-free synthesis of an oligonucleotide comprising: a first reaction chamber comprising a transferase; and a second reaction

chamber comprising a hydrolase, wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber, further comprising a third reaction chamber comprising a pyrophosphatase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the pyrophosphatase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the pyrophosphatase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the pyrophosphate is retained in the third reaction chamber comprising the pyrophosphatase.

[0200] In some aspects, the third reaction chamber comprises a ssRNA ligase wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the ssRNA ligase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the ssRNA ligase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the ssRNA ligase is retained in the third reaction chamber. In some aspects, the reaction chamber comprising the ssRNA ligase comprises a filter that prevents passage of ssRNA ligase and allows passage of the ligated oligonucleotide.

[0201] Thus in some aspects, provided herein is a system for template-free synthesis of an oligonucleotide comprising: a first reaction chamber comprising a transferase; and a second reaction chamber comprising a hydrolase, wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber, further comprising a third reaction chamber comprising a ssRNA ligase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the ssRNA ligase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the ssRNA ligase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the ssRNA ligase is retained in the third reaction chamber comprising the ssRNA ligase.

[0202] In some aspects, the third reaction chamber comprises a primase wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the primase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising

the primase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the primase is retained in the third reaction chamber.

[0203] Thus in some aspects, provided herein is a system for template-free synthesis of an oligonucleotide comprising: a first reaction chamber comprising a transferase; and a second reaction chamber comprising a hydrolase, wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber, further comprising a third reaction chamber comprising a primase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the primase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the primase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the primase is retained in the third reaction chamber comprising the primase. In some aspects, the reaction chamber comprising the primase comprises a filter that prevents passage of the primase and allows passage of a 3′ -blocked donor-acceptor oligonucleotide.

[0204] **FIG. 7** illustrates an example of a system in accordance with one implementation. The system comprises a first reaction chamber 700 comprising a transferase and a second reaction chamber 702 comprising a hydrolase that is fluidly connected by conduits 704, 706, and 708. Flow path is controlled through conduits 704, 706, and 708 by control valves 710 and 712. Second reaction chamber comprising a hydrolase 702 is fluidly connected back to first reaction chamber 700 via conduits 714, 716, and 718, and flow path is controlled by control valves 720 and 724. The flow is controlled by pump 726. The system further comprises a third reaction chamber comprising a second transferase 726. Third reaction chamber comprising a second transferase 726 is fluidly connected to first reaction chamber comprising a first transferase via conduits 704 and 728 through valve 710, and via conduits 730 and 718 through valve 724. Third reaction chamber comprising a second transferase 726 is fluidly connected to second reaction chamber comprising a hydrolase via conduits 706 and 708 through valve 712, and via conduits 814 and 816 through valves 720 and 724. Pump 726 is fluidly connected to the system using conduits 732 and 734. In some aspects, the enzyme in the third reaction chamber is a primase. In some aspects, the enzyme in the third reaction chamber is a ssRNA ligase.

[0205] In the system of **FIG. 7**, the oligonucleotide is introduced to the system by addition to first reaction chamber 700. In some implementations, the first reaction chamber comprises an apparatus for mixing solutions in first reaction chamber 700. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber comprising a transferase 700. The solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety is then flowed through conduit 704 into second reaction chamber comprising a hydrolase 702. The

solution is then flowed from the second reaction chamber 702 through conduits 714, 716 and 718 back to first reaction chamber comprising transferase. Alternatively, the solution can be flowed to the third reaction chamber comprising a second transferase via conduits 714 and 716 through valves 720 and 724. The movement of the solution, direction of the flow, and flow rate are parameters that can be controlled by optional components such as valves and pumps, such as control valves 720, 725, 710, 712, and pump 726. After each addition of nucleotide, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduit 804, 806, and 808 or 806 and 808 through control valves 728 or 728 and 712 into second reaction chamber 702.

[0206] The system may further comprise one or more reagent reservoirs. In some aspects, the system comprises a reagent reservoir comprising an inlet and an outlet; and wherein the system is configured to flow the oligonucleotide in the solution from an outlet of one of the reagent reservoir to the inlet of the first reaction chamber, from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from the outlet of the second reaction chamber to an inlet of the reagent reservoir. In some aspects, the reagent reservoir comprises an impeller. In some aspects, the reagent reservoir is replaceable or cleanable. In some aspects, the system further comprising a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the first reaction chamber. In some aspects, the product reservoir comprises an impeller. In some aspects, the product reservoir is replaceable or cleanable.

[0207] In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 700. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 700. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 700. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 702 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated oligonucleotide is then flowed through conduits 704 to first reaction chamber 700. In some implementations, the elongated oligonucleotide is flowed to first reaction chamber 700 and the hydrolase is retained in second reaction chamber 702. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 702. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 702. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or

support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 702. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to first reaction chamber 800 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into first reaction chamber 700, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 700 or second reaction chamber 702) in the system (e.g., into first reaction chamber 800 or second reaction chamber 702). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is

fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 700 and the second reaction chamber comprising the hydrolase 702). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 700 and the second reaction chamber comprising the hydrolase 702). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure

sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0208] **FIG. 8** is a system in accordance with one implementation. In the system, the reaction chamber comprising transferase 800 is fluidly connected to a second reaction chamber comprising hydrolase 802 by conduits 804 and 806 and valve 808. Second reaction chamber is fluidly connected back to first reaction chamber comprising transferase 800 by conduits 818 and 820 by valve 816. A reagent reservoir/ product reservoir 810 is also fluidly connected to the system by valves 822 and 816, and conduits 822 and 824.

[0209] In the system of **FIG. 8**, the oligonucleotide is introduced to the system by addition to first reaction chamber 800. In some implementations, the reservoir comprises an apparatus for mixing solutions in first reaction chamber 800. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber comprising a transferase 800. The solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety is then flowed through conduit 804 into second reaction chamber comprising a hydrolase 802. The solution is then flowed from the second reaction chamber 802 through conduits 818 or 806 back to the reagent reservoir which may optionally act as a product reservoir via control valves 816 or 822, respectively. From the reagent reservoir/ product reservoir, the solution can be cycled through to the first reaction chamber 800. The movement of the solution, direction of the flow, and flow rate are parameters that can be controlled by optional components such as valves and pumps, such as reagent reservoir 810. The first reaction chamber 800 comprises a transferase which conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduits 804 and 806. In some implementations, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduit 804 and 806 through control valve 808 into reagent reservoir 810 via conduit 822, mixed with new nucleotide comprising a 3′ blocking moiety, and then flowed into second reaction chamber 802 using control valve 816 through conduit 818, while transferase is retained in the first reaction chamber 800. **FIG. 9** is further embodiment of the system of FIG. 8, wherein there are one or more reagent reservoirs (e.g., shown here as 822-834, but may comprise more or less reagent reservoirs) optionally connected to reagent reservoir/product reservoir 810 by conduits 836 and 838. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 800. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 800. In some implementations the transferase is retained by immobilizing the

transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 800. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 802 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated oligonucleotide is then flowed through conduits 804 to first reaction chamber 800. In some implementations, the elongated oligonucleotide is flowed to first reaction chamber 800 and the hydrolase is retained in second reaction chamber 802. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 802. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 802. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 802. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to first reaction chamber 800 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into first reaction chamber 800, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 800 or second reaction chamber 802) in the system (e.g., into first reaction chamber 800 or second reaction chamber 802). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for

sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 800 and the second reaction chamber comprising the hydrolase 802). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 800 and the second reaction chamber comprising the hydrolase 802). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices,

memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0210] The system may further comprise a purification chamber. In some implementations, the system comprises a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from an oligonucleotide. In some implementations, the purification chamber comprises a column. In some implementations, the column is a liquid chromatography column. In some implementations, the purification chamber comprises size exclusion chromatography resin. In some implementations, the purification chamber comprises ion-exchange resin. In some implementations, the purification chamber comprises reverse phase resin. In some aspects, the purification chamber is configured to separate unreacted nucleotides or reaction byproducts in the solution from an oligonucleotide. In some aspects, the purification chamber is configured to capture incidental enzymes such as free transferase or free hydrolase that was not retained by the chamber.

[0211] **FIG. 10** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber 1010 and a second reaction chamber 1018. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. in the system, the reaction chambers are in line columns. Reagent reservoir 1000 is fluidly connected to a first reaction chamber 1010 comprising a transferase via conduits 1004 and 1008, optionally including pump 1006. First reaction chamber 1010 is fluidly connected to a second reaction chamber 1018 comprising a hydrolase by conduits 1012 and 1016, optionally an AT 1014 that is in line between first reaction chamber 1010. Second reaction chamber 1018 is fluidly connected to

purification chamber 1026 by conduits 1020 and 1024, optionally including AT 1022. Purification chamber 1028 is fluidly connected to product reservoir 1034 that comprises impeller 10106. Product reservoir 1034 is fluidly connected to reagent reservoir 1000 by conduit 1040. Product reservoir also is fluidly connected to conduit 10108. Optionally, in some implementations, the reagent reservoir 1000 and product reservoir are the same reservoir.

[0212] In the system of **FIG. 10**, the oligonucleotide is introduced to the system by addition into reagent reservoir 1000. In some implementations, the reservoir comprises an apparatus for mixing solutions in reagent reservoir 1000, such as impeller 1002. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to reagent reservoir 1000. Impeller 1002 is used to stir the solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety, then the liquid is flowed through conduits 1004 and 1008 via pump 1006 into a first reaction chamber 1010. The first reaction chamber 1010 comprises a transferase. The transferase conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduits 1012 and 1016, passing through AT 1014 into a second reaction chamber 1018. In some implementations, the elongated oligonucleotide comprising a 3′ blocking moiety is flowed through conduits 1012 and 1016, and into second reaction chamber 1018 and the transferase is retained in the first reaction chamber 1010. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 1010. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 1010. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 1010. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 1018 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. The elongated oligonucleotide is flowed through conduits 1020 and 1024, and optionally via AT 1012, and into purification chamber 1026. In some implementations, the elongated oligonucleotide is flowed to purification chamber 1026 and the hydrolase is retained in second reaction chamber 1018. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 1018. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 1018. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 1018. The in-line column comprises resin

or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. In some implementations, the elongated oligonucleotide is flowed through conduits 1020 and 1024, and into purification chamber 1026 and the hydrolase is retained in the second reaction chamber 1018. The elongated oligonucleotide is optionally purified in purification chamber 1026, then flowed through conduits 1028 and 1032, and optionally via AT 1030 into product reservoir 1034. Product reservoir 1034 comprises impeller 1036, which is used to stir the solution comprising the elongated oligonucleotide. The elongated oligonucleotide can exit the system using conduit 1038 or can be flowed to reagent reservoir 1000 through conduit 1040. Flowing the elongated oligonucleotide to reagent reservoir 1000 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into reagent reservoir 1000, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may also comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into reagent reservoir 1000, first reaction chamber 1010 or second reaction chamber 1018) in the system (e.g., into first reaction chamber 1010 or second reaction chamber 1018). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir.

In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1010 and the second reaction chamber comprising the hydrolase 1018). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1010 and the second reaction chamber comprising the hydrolase 1018). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. For example, the upon introduction into reagent reservoir 1000 of the system, the oligonucleotide is flowed through the system using a pre-determined flow rate until it reaches product reservoir 1034. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-

limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0213] **FIG. 11** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber 1114 and a second reaction chamber 1122. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. In the system, the reaction chambers are in line columns. Reagent reservoir 1100 comprises impeller 1102, and is fluidly connected to a first reaction chamber 1114 comprising a transferase via conduits 1104 and 1108, optionally including pump 1106. First reaction chamber 1114 is fluidly connected to a second reaction chamber 1122 comprising a hydrolase by conduits 1116, 1138, 1104, 1108, 1110, 1120, valves 1112, 1120, 1118, pump 1106, and reservoir 1110. Second reaction chamber 1122 is fluidly connected to purification chamber 1128 1116, 1138, 1104, 1108, 1110, 1120, 1126, 1136, valves 1112, 1120, 1118, 1132, pump 1106, and reservoir 1110. Product reservoir 1034 is fluidly connected to reagent reservoir 1000 by conduit 1040. Optionally, in some implementations, the reagent reservoir 1000 and product reservoir are the same reservoir.

[0214] In the system of **FIG. 11**, the oligonucleotide is introduced to the system by addition into reagent reservoir 1100. In some implementations, the reservoir comprises an apparatus for mixing solutions in reagent reservoir 1100, such as impeller 1102. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to reagent reservoir 1100. Impeller 1102 is used to stir the solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety, then the liquid is flowed through conduits 1104 and 1108 via pump 1106 into a diverter valve 1108. From the diverter valve, the solution is flowed through conduit 1110, through two diverter valves 1112 and 1120. The solution is routed to the first reaction chamber 1114 via conduit 1124, then flowed through conduit 1116 to reach another diverter valve 1118. Diverter valve 1118 flows the solution back to the reagent reservoir 1100 by conduit 1138, and further rerouted through conduits 1104, 1108, and diverter valve 1118. The first reaction chamber 1114 comprises a transferase. The transferase conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The

solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduits 1104, 1108, and 1110 through diverter valves 1112 and 1120 into a second reaction chamber 1122 via conduit 1128. Conduit 1130 routes the solution through diverter valve 1118 and conduit 1138 for return back to reagent chamber 1100. This cycle between first reaction and second reaction chamber can be reiterated any number of times. The oligonucleotide can optionally be purified between extension cycles or at the end of the reaction. From the reagent reservoir 1100, the solution is flowed through conduits 1104 and 1108 and 1110 by pump 1106 and through conduits 1122 and 1120 through a purification chamber 1128. Conduit 1130 flows the solution to diverter valve 1132, where the purified oligonucleotide can return to reaction chamber 1100 for further extension/ deblocking using conduit 1136, or exit the system using conduits 1134 and 1138 through diverter valve 1118. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 1114. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 1114. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 1114. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 1122 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 1122. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 1122. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 1122. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. The system optionally comprises solid bed reaction chambers. Flowing the elongated oligonucleotide to reagent reservoir 1100 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) The system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into reagent reservoir 1100, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations

(e.g., into reagent reservoir 1100, first reaction chamber 1114 or second reaction chamber 1122) in the system (e.g., into first reaction chamber 1114 or second reaction chamber 1122). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1114 and the second reaction chamber comprising the hydrolase 1122). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1114 and the second reaction chamber comprising the hydrolase 1122). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is

flowed through the system. In some implementations, the flow for the system is semi-automated. For example, the upon introduction into reagent reservoir 1100 of the system, the oligonucleotide is flowed through the system using a pre-determined flow rate until it exits the system. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0215] **FIG. 12** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber 1200 and a second reaction chamber 1214. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. First reaction chamber comprising a transferase is fluidly connected to second reaction

chamber 1214 comprising a hydrolase. First reaction chamber further comprises an impeller 1202 and second reaction chamber comprises impeller 1216. The first and second reaction chambers are fluidly connected by conduits 1204, 1208, 1210, 1218, 1222, 1218, 1224, 1243 and valves 1212, 1214, and pumps 1206, 1220. There is an optional purification column 1228 fluidly connected to the first and second reaction chambers by conduits 1226 and 1230 and via valves 1212 and 1224.

[0216] In the system of **FIG. 12**, the oligonucleotide is introduced to the system by addition into reagent reservoir 1200. In some implementations, the reservoir comprises an apparatus for mixing solutions in reagent reservoir 1200, such as impeller 1202. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber 1200. An optional impeller, such as Impeller 1202 is used to stir the solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety, then the liquid is flowed through conduits 1204 and 1208 via pump 1206 into a diverter valve 1208. From the diverter valve, the solution is flowed through conduit 1210, through diverter valve 1212 into a second reaction chamber 1214, which also comprises an optional impeller 1316. The first reaction chamber 1200 comprises a transferase. The transferase conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduits 1204, 1208 through pump 1206 and diverter valve 1212 into a second reaction chamber 1214. Conduits 1218 and 1222 and 1232 route the solution back to the first reaction chamber 1200. This cycle between first reaction and second reaction chamber can be reiterated any number of times. The oligonucleotide can optionally be purified between extension cycles or at the end of the reaction by routing the solution to the first reaction chamber as described above, flowing the solution through conduits 1204 and 1208 through pump 1206, and using diverter valve 1212 to flow the solution through purification chamber 1228. Conduit 1230 is used to connect the purification chamber to diverter valve 1224, which cycles the solution to the first reaction chamber for more rounds of extension/ deblocking, purification, or collecting the product (e.g., using the first reaction chamber as a product reservoir). In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 1200. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 1200. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 1200. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 1214 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of

second reaction chamber 1214. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 1214. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 1214. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to reagent reservoir 1200 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into reagent reservoir 1200, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 1200 or second reaction chamber 1214) in the system (e.g., into first reaction chamber 1200 or second reaction chamber 1214). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically

conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1200 and the second reaction chamber comprising the hydrolase 1214). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1200 and the second reaction chamber comprising the hydrolase 1214). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. For example, upon introduction into reagent reservoir 1300 of the system, the oligonucleotide is flowed through the system using a pre-determined flow rate until it exits the system. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated

oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0217] In some aspects, the system is configured for template-free synthesis of an oligonucleotide comprising: a reagent reservoir; a first column configured to substantially separate a transferase from an oligonucleotide comprising a 3′ blocking moiety; and a second column configured to substantially separate a hydrolase from an oligonucleotide without the 3′ blocking moiety; wherein the system is configured to (i) flow a solution comprising an oligonucleotide comprising the 3′ blocking moiety and a transferase from the reagent reservoir to the first column, (ii) substantially separate the oligonucleotide comprising the 3′ blocking moiety from the transferase in the first column, (iii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety from the first column to the reagent reservoir, (iv) flow the solution comprising an oligonucleotide without the 3′ blocking moiety and a hydrolase to the second column, (v) substantially separate the oligonucleotide without the 3′ blocking moiety from the hydrolase in the second column, and (vi) flow the solution comprising the oligonucleotide without the 3′ blocking moiety from the second column to the reagent reservoir. In some aspects, the system comprises a plurality of conduits that connects the first column, the second column, and the reagent reservoir. In some aspects, the system comprises a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety.

[0218] **FIG. 13** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 1300 that acts as a second reaction chamber that comprises a hydrolase, wherein the chamber optionally comprises impeller 1302. In this system, the chamber comprises one enzyme at a time. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. The reaction chamber is fluidly connected to a series of purification chambers 1320, 1326, 1332 to remove transferase, hydrolase, and/or to purify the oligonucleotide. These purification chambers are fluidly connected to reaction chamber 1300 by conduits 1304, 1308, 1316, 1318, 1324, 1330, 1322, 1328,

1340, 1334, 1344, and through valves 1319, 1314, 1338, 1336, 1342. The flow is controlled by pump 1306. The system is also fluidly connected to reagent chamber 1318.

[0219] More specifically, reaction chamber 1300 comprising a transferase is fluidly connected to a purification column that removes transferase 1320 via conduits 1304, 1308, 1352, 1318, 1322, 1342, 1344, and 1346. The flow is driven by pump 1306 and the flow path is regulated by diverter valves 1310, 1314, 1336, and 1344. As depicted in this system, the transferase can optionally be TdT.

[0220] Reaction chamber 1300 comprising a hydrolase is fluidly connected to a purification column that removes hydrolase 1326 via conduits 1304, 1308, 1352, 1324, 1328, 1342, 1344, and 1346. The flow is driven by pump 1306 and the flow path is regulated by diverter valves 1310, 1314, 1336, and 1344. As depicted in this system, the hydrolase can optionally be alkaline phosphatase.

[0221] Reaction chamber 1300 can also act as a product reservoir, and is fluidly connected to a purification column that purifies the oligonucleotide 1332 via conduits 1304, 1308, 1352, 1354, 1330, 1334, 1340, 1342, 1344, and 1346. The flow is driven by pump 1306 and the flow path is regulated by diverter valves 1310, 1314, 1336, 1338, and 1344.

[0222] The system comprises a reagent reservoir 1318, which optionally comprises wash buffer(s). Reagent reservoir 1318 is replaceable or cleanable. Reagent reservoir 1318 is fluidly connected to the three purification chambers 1320, 1326, and 1332 via conduits 1316, 1352, and 1324. The flow path is regulated by valve 1314.

[0223] In the system of **FIG. 13**, the oligonucleotide is introduced to the system by addition into a first reaction chamber 1300. In this particular embodiment, the first reaction chamber and the second reaction chamber are the same chamber. In this system, the chamber comprises one enzyme at a time. The solution is mixed, optionally using impeller 1302, then flowed through conduits 1304 and 1308 using pump 1306 to diverter valve 1310. To remove the transferase, the solution is flowed through diverter valve 1324 through conduit 1318 into a column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety 1320. The solution is flowed through conduit 1322 to diverter valve 1336, and further through conduits 1342 and 1346 to reroute the solution back to the first reaction chamber 1300. A hydrolase is then added to the first reaction chamber 1300. In some implementations, the reservoir comprises an apparatus for mixing solutions in reagent reservoir 1300, such as impeller 1302. Preselected nucleotide (e.g., a nucleotide with a 3′ blocking moiety such as a NQP) can also be added to first reaction chamber 1300. An optional impeller, such as Impeller 1302 is used to stir the solution comprising the oligonucleotide and the nucleotide comprising a 3′ blocking moiety, then the liquid is flowed through conduits 1304 and 1308 via pump 1306 into a diverter valve 1308. From the diverter valve, the solution is flowed through conduit 1310, through diverter valve 1312 into a second reaction chamber 1314, which also comprises an optional impeller 1316. The first reaction chamber 1300 comprises a transferase. The transferase

conjugates the nucleotide comprising a 3′ blocking moiety to the oligonucleotide. The solution, now comprising an elongated oligonucleotide comprising a 3′ blocking moiety is then flowed through conduits 1304, 1308 through pump 1306 and diverter valves 1310 and 1314 to remove the hydrolase via column 1326. The solution is flowed through conduits 1328, 1344 and 1346 to return to first reaction chamber 1300. This cycle between first reaction and second reaction chamber can be reiterated any number of times. The oligonucleotide can optionally be purified between extension cycles or at the end of the reaction by routing the solution to the first reaction chamber as described above, flowing the solution through conduits 1304 and 1308 through pump 1306, and using diverter valves 1310 and 1314 to flow the solution through purification chamber 1432. Conduit 1434 is used to connect the purification chamber to diverter valve 1338, which cycles the solution to the first reaction chamber 1300 through valves 1436, and conduits 1340, 1342, 1344, and 1346 for more rounds of extension/ deblocking, purification, or collecting the product (e.g., using the first reaction chamber as a product reservoir). Optionally, the reagent may leave the system post purification via diverter valve 1438 and conduit 1448. In some implementations, the transferase is retained by a membrane positioned near the outlet of first reaction chamber 1400. In some implementations, the transferase is retained by a membrane at the outlet of first reaction chamber 1400. In some implementations the transferase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the transferase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the first reaction chamber 1300. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Second reaction chamber 1314 comprises a hydrolase, which is used to remove the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to produce an elongated oligonucleotide. In some implementations, the hydrolase is retained by a membrane positioned near the outlet of second reaction chamber 1314. In some implementations, the hydrolase is retained by a membrane at the outlet of second reaction chamber 1314. In some implementations the hydrolase is retained by immobilizing the transferase on a matrix or support, such as a bead or resin. In some implementations, the hydrolase that is incidentally released may be captured by an in-line column positioned downstream near the outlet of the second reaction chamber 1314. The in-line column comprises resin or beads that can be used for capturing transferase, such as but not limited to affinity capture (e.g., nickel or cobalt resin, affinity tags, antibodies) and ion exchange. Flowing the elongated oligonucleotide to reagent reservoir 1300 reintroduces an oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into reagent reservoir 1300, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as

described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 1300) in the system (e.g., into first reaction chamber 1300). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1300 and the second reaction chamber comprising the hydrolase 1314). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1300 and the second reaction chamber comprising the hydrolase 1314). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional

reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. For example, the upon introduction into reagent reservoir 1300 the system, the oligonucleotide is flowed through the system using a pre-determined flow rate until it exits the system. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0224] **FIG. 14** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 1400 and a second reaction chamber that comprises a hydrolase 1402. Reservoirs and reaction

chambers can be, for example, batch vessels, flasks, or in line columns. First reaction chamber 1400 is fluidly connected to reagent reservoir/ product reservoir 1404 by conduits 1414 and 1418. Conduits 1414 and 1418 are fluidly connected to a series of in-line meters or detectors 1416. Optional in-line meters or detectors include flow meter 1442, UV-Vis detector 1444, conductivity meter 1446 and pH meter 1448. Reagent reservoir/ product reservoir 1404 comprises optional impeller 1438 and is fluidly connected to first reaction chamber 1400 by conduits 1406, 1410, and 1452 and comprise in-line pump 1408 to flow the fluid through the conduits, and an in-line degasser 1412 and a pressure meter 1440. Reagent reservoir/ product reservoir 1404 is also fluidly connected to a second reaction chamber comprising a hydrolase 1402 via conduit 1440 which optionally comprises pump 1422, in-line degasser 1424 and pressure meter 1454. The second reaction chamber comprising a hydrolase 1402 is fluidly connected to a reagent reservoir/ product reservoir 1434 comprising optional impeller 1436 via conduits 1450 and 1432. Optional meters such as a UV-Vis detector 1428 and pressure meter 1430 are included between conduits 1450 and 1432.

[0225] In the system depicted in **FIG. 14**, the oligonucleotide is introduced to the system by addition into reagent reservoir/product reservoir 1404, which comprises optional impeller 1438. The oligonucleotide is flowed through conduits 1406, 1410, and 1452 by pump 1408, passing through degasser 1412 and pressure meter 1440, and enters first reaction chamber comprising a transferase 1400. In this exemplary system, the oligonucleotide is elongated at 40 °C to produce an oligonucleotide comprising a 3′ blocking moiety, then travels through conduit 1414 and 1418, passing through optional detectors such as flow meter 1442, UV-Vis detector 1444, conductivity meter 1446, and pH meter 1448 into a reagent reservoir/ product reservoir 1404 comprising optional impeller 1438. In **FIG. 14**, reagent reservoir/ product reservoir 1404 is a jacketed stir tank which is optionally maintained between 2 °C to 8 °C, such as at 4 °C. The oligonucleotide comprising the 3′ blocking moiety is then flowed through conduit 1420 by pump 1422 through an in-line degassing unit 1424 and pressure meter 1454, through conduit 1426 and into second reaction chamber comprising a hydrolase 1402. In **FIG. 14**, second reaction chamber comprising a hydrolase 1402 is a packed bed reactor comprising the hydrolase such as alkaline phosphatase. The oligonucleotide comprising a 3′ blocking moiety is deblocked at 50 °C to produce an elongated oligonucleotide, which travels through conduits 1450 and 1432, through UV-Vis detector 1428 and pressure meter 1430, to reagent reservoir/ product reservoir 1434 comprising optional impeller 1436. In **FIG. 14**, reagent reservoir/product reservoir 1434 is a jacketed stir tank which can be optionally maintained between 2 °C to 8 °C, such as at 4 °C. The elongated nucleotide can then be collected from reagent reservoir/ product reservoir 1434 and reintroduced into reagent reservoir product reservoir 1404, which re-enters the elongated nucleotide into the system. The system as described in **FIG. 14** can be reiterated such that each iteration further elongates the oligonucleotide by one nucleotide until an oligonucleotide of a specified sequence is made. Flowing the elongated oligonucleotide to reagent reservoir 1404 reintroduces an

oligonucleotide into the system. Upon introduction of another preselected nucleotide (e.g., a second nucleotide, a second nucleotide with a 3′ blocking moiety such as a NQP) the system can be reiterated to elongate the oligonucleotide such that each iteration further elongates the oligonucleotide by one nucleotide. In some implementations, upon entry into reagent reservoir 1404, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 1400) in the system (e.g., into first reaction chamber 1400). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1400 and the second reaction chamber comprising the hydrolase 1402). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3′-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is

fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1400 and the second reaction chamber comprising the hydrolase 1402). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. For example, upon introduction into the system via reagent reservoir/product reservoir 1404, the oligonucleotide is flowed through the system using a pre-determined flow rate until it exits the system. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some

implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0226] **FIG. 15** illustrates an example of a system in accordance with one implementation. The system is an example of a system that comprises a first reaction chamber comprising a transferase 1500 and optional impeller 1508 and a second reaction chamber that comprises a hydrolase 1502 and optional impeller 1524. Reservoirs and reaction chambers can be, for example, batch vessels, flasks, or in line columns. First reaction chamber 1500 is fluidly connected to reagent reservoir/ product reservoir 1506 by conduit 1504. Reagent reservoir/ product reservoir 1506 is fluidly connected to synthesizer 1512 which comprises pH meter 1514, conductivity meter 1516, and UV-Vis detector 1518. Synthesizer 1512 is fluidly connected to first reaction chamber comprising a transferase 1500 via conduit 1540, to desalting chamber 1530 via conduit 1528, and to second reaction chamber comprising a hydrolase 1502 via conduit 1520. Synthesizer 1512 is also fluidly connected to reagent reservoirs comprising NQPs and buffers, such as reagent reservoirs 1534 and 1536. Second reaction chamber comprising a hydrolase 1502 is fluidly connected to reagent reservoir/product reservoir 1538 comprising impeller 1524, which is fluidly connected to the synthesizer via conduit 1526. Desalting chamber 1530 is fluidly connected to reagent reservoir/ product reservoir 1538 via conduit 1524.

[0227] In the system depicted in **FIG. 15**, the oligonucleotide is introduced to the system by addition into reagent reservoir/product reservoir 1506 or synthesizer 1512. In one exemplary embodiment of the systems described herein, the system comprises a first reaction chamber comprising a transferase 1500 and a second reaction chamber comprising a hydrolase 1502. In this exemplary system, the oligonucleotide is elongated at 40 °C to produce an oligonucleotide comprising a 3′ blocking moiety, then travels through conduit 1504 into a reagent reservoir/ product reservoir 1506, optionally comprising impeller 1508. Reagent reservoir/ product reservoir 1506 can be a jacketed stir tank, which is optionally maintained between 2 °C to 8 °C. The oligonucleotide comprising the 3′ blocking moiety is then flowed through bidirectional conduit 1510 and travels through synthesizer 1512 comprising optional meters that can be used to monitor the conditions of the system. In this exemplary system, the meters or detectors include pH meter 1514, conductivity meter 1516 and UV-Vis detector 1518. The oligonucleotide comprising the 3′ blocking moiety is flowed into second reaction chamber comprising a hydrolase 1502. The oligonucleotide comprising the 3′ blocking moiety is deblocked to produce an elongated oligonucleotide, then the elongated oligonucleotide is flowed via conduit 1522 into a second reagent chamber/ product reservoir 1538 comprising impeller 1524. The elongated oligonucleotide is flowed via conduit 1526 to synthesizer 1512, where it flows through pH meter 1514, UV-Vis detector 1518, and conductivity meter 1516. The elongated oligonucleotide travels through conduit 1528 to desalting chamber 1530, where the oligonucleotide is separated, and buffer exchanged. The elongated oligonucleotide travels via conduit 1532 into reagent reservoir/ product reservoir 1526. The elongated oligonucleotide can either be collected from reagent reservoir/ product

reservoir 1526, or rerouted through conduit 1520, through synthesizer 1512 and its meters to re-enter the first reaction chamber comprising a transferase 1500 via conduit 1540, where the elongated oligonucleotide is further elongated by the transferase to produce a oligonucleotide comprises a 3′ blocking moiety. The oligonucleotide comprising a 3′ blocking moiety can be flowed through the system as described in **FIG. 15**, and the cycles can be reiterated such that each iteration further elongates the oligonucleotide by one nucleotide until an oligonucleotide of a specified sequence is made. In some implementations, upon entry into reagent reservoir 1506, the elongated oligonucleotide becomes an oligonucleotide that is introduced into the system. In some implementations, the conduits, reservoirs and/or chambers as described above in the system may comprise one or more ports, such as an injection port that can be used to introduce additional reagents (e.g., predetermined nucleotide substrate, buffers, reagents, and/or cofactors) at defined locations (e.g., into first reaction chamber 1500) in the system (e.g., into first reaction chamber 1500). Optionally, the system described herein comprises additional detectors, valves, or pumps that are configured in line with the conduits, and permit fluid connection between the chambers and/or reservoirs. For example, the system can have one or more pumps, one or more UV detectors, one or more degassers, or one or more valves integrated between the conduits in a manner that permits fluid connections between the chambers and/or reservoirs. In some aspects, the degasser can be integrated downstream of a reagent reservoir comprising buffers and/or reagents such that the degasser is fluidly connected to the system, and the buffer and/or reagents are degassed prior to introduction into the system. In some aspects, the degasser is an in-line degasser that is fluidly connected to the system, and the solution is degassed as it is flowed through the system. In some aspects, the system may include a reservoir that is fluidly connected to a source of inert gas for sparging the contents of the reservoir. In some aspects, the system may comprise one or more in-line spargers for sparging the solutions within the system. In some aspects, the system described herein may comprise one or more purification chambers. The systems described herein may comprise two purification chambers. In some aspects, the purification chambers are different types of purification chambers. As a non-limiting example, the system described herein may also comprise at least one purification chamber (e.g., an enzyme capture chamber) and at least one desalting chamber that can be used to separate, purify, buffer exchange and/or concentrate the oligonucleotide. Exemplary placements of a desalting chamber in the systems and methods include, but are not limited to, before or after a reaction chamber or a product reservoir. In some aspects, the oligonucleotide is first desalted and concentrated using a desalting chamber, then flowed into a reaction chamber, where the oligonucleotide is enzymatically conjugated to a conjugation moiety. In some aspects, the enzymatic conjugation reaction can be performed in batch. In some aspects, the system comprises an additional reaction chamber comprising a primase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1500 and the second reaction chamber comprising the hydrolase 1502). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide

acceptor having a 3′-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the system comprises an additional reaction chamber comprising a ssRNA ligase that is fluidly connected to the system disclosed herein (e.g., the first reaction chamber comprising the transferase 1500 and the second reaction chamber comprising the hydrolase 1502). In some aspects, the additional chamber comprises a ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the additional reaction chamber comprises a solid support or filter to retain the primase or ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system. In some implementations, the flow for the system is semi-automated. For example, upon introduction into the system via reagent reservoir/product reservoir 1506 or via a valve or port that is fluidly connected to synthesizer 1512, the oligonucleotide is flowed through the system using a pre-determined flow rate until it exits the system. In some implementations, the flow for the system is automated. In some implementations, the flow can be controlled by a device, such as a flow controller, one or more valves, one or more pumps, or one or more flow meters. The flow rate can be manually or automatically adjusted. In some implementations, the flow rate is adjusted by a device, such as a device that is connected to the system. In some implementations, the system is connected to a device. In some implementations, the flow rate can be adjusted by a device that is connected to the system. In some implementations, the device is a host computer connected to a network. In some implementations, the host computer is a client computer or a server. In some implementations, the device can be any suitable type of microprocessor-based device, such as a personal computer, workstation, server or handheld computing device (portable electronic device) such as a phone or tablet. The device can include, for example, one or more processor(s), input devices, output devices, memory or storage devices, communication devices, and one or more analytical systems for (for example, one or more liquid chromatography systems and/or one or more mass spectrometers). Software that resides in the memory or storage device may comprise, for example, an operating system and software for controlling the system (e.g., a synthesizer), such as controlling automation of the system or controlling iterative cycles of the system. In some implementations, the system is connected to an analytical system. The analytical system can be used to assess properties of the oligonucleotide that is flowed through the system. Non-limiting examples include quantity of elongated oligonucleotide, sequence of the elongated oligonucleotide, mass of the elongated oligonucleotide and/or oligonucleotide comprising a 3′ blocking moiety, purity of the elongated oligonucleotide, and the presence and/or quantity of any byproducts, such as the byproducts disclosed herein, generated by flowing the oligonucleotide through the system. Non-limiting examples of analytical systems include UV detectors, mass spectrometers, chromatography systems. The system may further comprise sensors such as pressure sensors, pH meters, conductivity meters, to monitor the

conditions of the system or each individual reservoir or chamber. In some implementations, the sensors are connected in line with the conduits such that the sensor is measuring the solution that is flowing, such as flowing in conduits, or flowing between reservoirs and/or chambers. In some implementations, the sensors are in contact with the interior of the reservoirs or chambers of the system to monitor the conditions and/or detect changes in the solution.

[0228] In some aspects, a fraction of the bound or immobilized enzyme (e.g., the transferase or the hydrolase, or pyrophosphatase) may be stripped or may disengage from the solid support, or pass through the filter or membrane along with the elongated oligonucleotide comprising a 3′ blocking moiety, or the elongated oligonucleotide. In some aspects, the system may further comprise one or more in-line purification chambers to be used for removing any contaminating or trace levels of enzyme that exits the reaction chambers along with the nucleic acid (e.g., an oligonucleotide, an elongated oligonucleotide, or an elongated oligonucleotide comprising a 3′ blocking moiety). In some aspects, the in-line chamber is downstream of the first reaction chamber. In some aspects, the in-line chamber is immediately downstream of the first reaction chamber. In some aspects, the in-line chamber is downstream of the second reaction chamber. In some aspects, the in-line chamber is immediately downstream of the second reaction chamber. In some aspects, the in-line chamber is a purification chamber. In some aspects, the in-line chamber comprises affinity resin, ion exchange resin, or other resin or matrices that are known to separate molecules and macromolecules in solution based on their inherent proteins. In some aspects, the in-line chamber comprises a resin that captures the histidine tag. In some aspects, the resin is nickel, cobalt, or immobilized metal affinity chromatography (IMAC). In some aspects, the system may further comprise additional filters or membranes to be used for removing any contaminating or trace levels of enzyme that exits the reaction chambers along with the nucleic acid (e.g., an oligonucleotide, an elongated oligonucleotide, or an elongated oligonucleotide comprising a 3′ blocking moiety).

Methods of Template-Free Synthesis of an Oligonucleotide

[0229] The present disclosure provides for methods of template-free synthesis of an oligonucleotide, and methods that use the system described herein for template-free synthesis of an oligonucleotide.

[0230] Any of the methods disclosed herein for template-free synthesis of an oligonucleotide 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. Additional techniques known to one skilled in the art may be carried out to supplement the reaction conditions. These can include taking measures to stabilize or prevent inactivation of the enzyme, reduce product inhibition, shift reaction equilibrium to formation of the desired product.

[0231] In some aspects, the methods disclosed herein synthesizes the oligonucleotide to be elongated by the transferase using a primase contained in an additional reaction chamber that is fluidly connected to the system (e.g., first reaction chamber and the second reaction chamber). In some aspects, the oligonucleotide is synthesized in a template independent manner comprising reacting a nucleotide acceptor having a 3'-OH group and a nucleotide donor in presence of a primase in the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the primase is retained in the additional reaction chamber by a solid support or filter to retain the primase in the additional reaction chamber while the oligonucleotide is flowed through the system.

[0232] In some aspects, the methods provided herein further comprises ligating an oligonucleotide acceptor with a nucleotide donor (e.g., a single nucleotide donor or an oligonucleotide donor) using a ssRNA ligase. In some aspects, the ssRNA ligase that ligates the oligonucleotide synthesized in a template independent manner using the methods and systems system described herein to another nucleotide or oligonucleotide using the ssRNA ligase contained within the additional reaction chamber that is fluidly connected to the system described herein. In some aspects, the ssRNA ligase is retained in the additional reaction chamber by a solid support or filter to retain the ssRNA ligase in the additional reaction chamber while the oligonucleotide is flowed through the system.

[0233] In some aspects, the methods provided herein include synthesizing oligonucleotides without one or more impurities (e.g., unintentionally modified oligonucleotides). Oligonucleotide synthesis using enzyme mediated elongation and deblocking has advantages of avoiding impurities generated by phosphoramidite chemistry oligonucleotide synthesis, such as product related impurities that arise from chemically mediated oligonucleotide synthesis. Because the downstream application oligonucleotides are commercial or therapeutic in nature, impurities that compromise oligonucleotide quality (such as, but not limited to oligonucleotide degradation or oligonucleotides that are unintentionally modified such as chemically modified, side-reaction modified, or degraded oligonucleotides) are undesirable. In some aspects, the one or more impurities may be impurities that are generated during chemical synthesis of oligonucleotides. In some aspects, the impurity is a depurinated oligonucleotide. Depurination is the loss of a purine base from a nucleotide backbone, resulting in an unintended abasic nucleotide. In some aspects, the impurity is a depyrimidated oligonucleotide. Depyrimidation is the loss of a pyrimidine base from a nucleotide backbone, resulting in an unintended abasic nucleotide. In some aspects, the impurity is an oligonucleotide comprising a c-Net modification. cNet modified oligonucleotides comprise a N3-(2-cyanothethy)thymine in place of an intended thymine nucleotide. In some aspects, the impurity is an oligonucleotide comprising a methyl group (e.g., a methylated oligonucleotide). In some aspects, the methyl group is on a cytosine such that the impurity is an oligonucleotide comprising a methylcytosine in place of an intended cytosine nucleotide. In some aspects, impurity is an

oligonucleotide comprising an acetyl or isobutyryldiaminopurine (IBU) in place of an intended guanine.

[0234] In some aspects, the composition comprising the oligonucleotide made with any of the systems or methods described herein is substantially free of one or more impurities. In some aspects, the composition comprising the oligonucleotide made by the methods or systems described herein is substantially free of the identified impurity, such that the composition comprises about 5% or less, about 4% or less, about 3% or less, about 2% or less, about 1% or less, about 0.5% or less, about 0.25% or less or about 0.1% or less of the identified impurity. In some aspects, the composition comprising the oligonucleotide made by the methods described herein is substantially free of the identified impurity, such that the composition comprises 5% or less, 4% or less, 3% or less, 2% or less, 1% or less, 0.5% or less, 0.25% or less or 0.1% or less of the identified impurity. In some aspects, the composition comprising the oligonucleotide made by the methods described herein is substantially free of the identified impurity, such that the composition comprises about less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.25%, or less than 0.1% of the identified impurity. In some aspects, the identified impurity is not detectable in the composition. In some aspects, the oligonucleotide is substantially free of depurination or depyrimidination impurities. In some aspects, the oligonucleotide is substantially free of N-3-cyanoethylthymine (CNET) impurities. In some aspects, the oligonucleotide is substantially free of N(2)-actyl-2,6-diaminopurine and/or an isobutyryl diaminopurine impurities. In some aspects, the oligonucleotide is substantially free of methylcytosine. In some embodiments, the composition comprising the oligonucleotide synthesized using the method described herein have less than 30%, 25%, 20%, 15%, 10%, 5%, or 2% or less of phosphorothioate internucleoside linkages converted to the corresponding phosphodiester linkages. In some embodiments, the composition comprising the oligonucleotide synthesized using the methods described herein is substantially free of Class IV impurities, as described in Capaldi et al., Nucl Acid Ther., 2017, 27(6):309, incorporated by reference herein. In some embodiments, the composition of the synthesized oligonucleotide has no detectable Class IV impurities under standard assay conditions. As described in Capaldi et al., Class IV impurities contain "structural elements not found in the parent oligonucleotide or in naturally occurring nucleic acids, and impurities of unknown structure." (see, e.g., Table 1 of Capaldi et al., incorporated by reference herein).

[0235] In some aspects, template free synthesis of an oligonucleotide can be carried out at about 30 °C to about 95 °C. In some embodiments, the reaction temperature is about 35 °C to about 90 °C, about 40 °C to about 85 °C, about 45 °C to about 80 °C, about 50 °C to about 75 °C, or about 55 °C to about 70 °C. In some embodiments, the suitable reaction conditions for the reaction with the recombinant primase comprises a temperature of about 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.

[0236] In some embodiments, the suitable reaction conditions for the reaction with the transferase comprises a temperature of about 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 suitable reaction conditions for the reaction with the transferase comprises a temperature of 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 suitable reaction conditions for the reaction with the transferase comprises a temperature of 40 °C.

[0237] In some embodiments, the suitable reaction conditions for the reaction with the inorganic pyrophosphatase comprises a temperature of about 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 suitable reaction conditions for the reaction with the inorganic pyrophosphatase comprises a temperature of 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 suitable reaction conditions for the reaction with the inorganic pyrophosphatase comprises a temperature of 40 °C.

[0238] In some embodiments, the suitable reaction conditions for the reaction with the hydrolase comprises a temperature of about 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 suitable reaction conditions for the reaction with the hydrolase comprises a temperature of 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 suitable reaction conditions for the reaction with the hydrolase comprises a temperature of 50 °C.

[0239] In some aspects, the methods of template free synthesis described herein can be carried out using buffers and/or solutions are degassed before or during operation of the systems as described herein.

[0240] In some aspects, the methods of template free synthesis described herein can be carried out using buffers and/or solutions are sparged before or during operation of the systems as described herein. Sparging adds a desired gas to the buffer, which saturates the buffer with the desired gas at the exclusion of an undesired gas.

[0241] The buffers and/or solutions may include reagents, such as reducing agents or antioxidants, that prevent or minimize oxidation. In some aspects, the antioxidant is used at a final concentration of about 2 mM to about 10 mM, about 1 mM to about 5 mM, about 0.5 mM to about 8 mM, about 30-80 mM, about 10-90 mM, about 40-70 mM, or about 0-100 mM. In some aspects, the antioxidant is used at a final concentration of about 0 mM, about 0.5 mM, about 1 mM, about 5 mM, about 10 mM, about 15 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, or about 100 mM. In some aspects, the antioxidant is used at 0 mM, 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, or 100 mM. In some embodiments, the antioxidants or reducing agents can be at a higher concentration, such as from

about 100 mM to about 500 mM, from about 150 to 450 mM, from about 200 mM to about 400 mM. In some embodiments, the antioxidant or reducing agent can be about 100 mM, 150 mM, 200 mM, 300 mM, 400 mM, or 500 mM. In some aspects, the antioxidant is used at a final concentration of 5 mM. In some aspects, the antioxidant is ascorbic acid, citric acid, formic acid, sodium thiosulfate, metabisulfite, 2,6-dimethoxyphenol, or catalase. In some aspects, the antioxidant is ascorbic acid, citric acid, formic acid, sodium thiosulfate, metabisulfite, or 2,6-dimethoxyphenol. In some aspects, the antioxidant is sodium thiosulfate.

[0242] In some aspects, the antioxidant is sodium thiosulfate. In some aspects, sodium thiosulfate is used at a final concentration of about 2 mM to about 10 mM, about 1 mM to about 5 mM, about 0.5 mM to about 8 mM, about 30-80 mM, about 10-90 mM, about 40-70 mM, or about 0-100 mM. In some aspects, sodium thiosulfate is used at a final concentration of about 0 mM, about 0.5 mM, about 1 mM, about 5 mM, about 10 mM, about 15 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, or about 100 mM. In some aspects, sodium thiosulfate is used at 0 mM, 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, or 100 mM. In some aspects, sodium thiosulfate is used at a final concentration of 5 mM.

[0243] In some aspects, the antioxidant is catalase. An example of use of the catalase in the methods as described herein is for the disproportionation of hydrogen peroxide. In some aspects, the methods comprise about 3000 units-7000 units, about 2000-6000 units, or about 1000-8000 units of catalase. In some aspects, the methods comprise about 3000 units, about 4000 units, about 5000 units, about 6000 units, or about 7000 units of catalase. In some aspects, the methods comprise 3000 units, about 4000 units, about 5000 units, about 6000 units, or about 7000 units of catalase. In some aspects, the methods comprise 5600 units (0.7 mg/ml) of catalase.

[0244] Template free synthesis can also be carried out at a pH suitable for the activities of any of the enzymes as disclosed herein. The pH can be about 6-9, pH of about 6.5-8.5, or pH of about 7-8. In some aspects, the pH of the reaction can be about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, or about 9. In some aspects, the pH of the reaction is 6, 6.5, 7, 7.5, 8, 8.5, or 9.

[0245] In some aspects, the residence time of each cycle is concentration-dependent on the oligonucleotide. Residence time is the amount of time a fluid is inside of a vessel, such as the chambers or reservoirs as described herein. In some aspects, the extension residence time is concentration dependent. In some aspects, the extension residence time is about 0.1, about 0.2, about 0.3 hours. In some aspects, the extension residence time is 0.1, 0.2, or 0.3 hours. In some aspects, the extension residence time is 0.1 hours. In some aspects, the deblocking residence time is about 0.1, about 0.2, about 0.3 hours. In some aspects, the deblocking residence time is 0.1, 0.2, or 0.3 hours. In some aspects, the deblocking residence time is 0.1 hours.

[0246] In some aspects, the reaction chamber is a column. In some aspects, the column comprises 3 grams of resin. In some aspects, the first reaction chamber comprising a hydrolase comprises 3 grams of resin. In some aspects, the second reaction chamber comprising a transferase comprises 3 grams of resin.

[0247] In further aspects, any of the above-described methods for template free-synthesis of an oligonucleotide can further comprise one or more steps selected from: extraction; isolation; or purification of the oligonucleotide. 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. Provided herein are methods of template-free synthesis of an oligonucleotide, comprising: elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase; removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed.

[0248] In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

[0249] In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase, such as a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase.

[0250] In some aspects, the method further comprises separating the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase. Separating (e.g., substantially separating) can be done by immobilizing the hydrolase on a solid support, or using a filter or membrane to entrap or prevent passage of the hydrolase while the oligonucleotide flows through the pores. In some aspects, the filter or membrane has a MWCO of a pre-determined value.

[0251] In some aspects, the method further comprises repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. The one or more cycles can be repeated at least once, at least twice, at least three, at least four, at least five, at least six times.

[0252] In some aspects, the method further comprises using a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety. An oligonucleotide of a

predetermined sequence may be generated by single addition of each pre-determined nucleotide to achieve said sequence.

[0253] In some aspects, the method further comprises using at least two different types of transferases in in separate cycles. Some transferases are better optimized to conjugate or ligate certain modified nucleotides to the oligonucleotide, due to reasons such as steric considerations that influence binding and/or efficient ligation.

[0254] In some aspects, the method comprises elongating the oligonucleotide in a first reaction chamber.

[0255] In some aspects, the method comprises removing the 3′ blocking moiety in a second reaction chamber.

[0256] Also provided herein are methods of template-free synthesis of an oligonucleotide, comprising: in a first reaction chamber, elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber, wherein the transferase is retained in the first reaction chamber; in the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed; and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the second reaction chamber into the first reaction chamber.

[0257] In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

[0258] In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase, such as a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase.

[0259] In some aspects, the method further comprises separating the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase. Separating (e.g., substantially separating) can be done by immobilizing the hydrolase on a solid support, or using a filter or membrane to entrap or prevent passage of the hydrolase while the oligonucleotide flows through the pores. In some aspects, the filter or membrane has a MWCO of a pre-determined value.

[0260] In some aspects, the method further comprises repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. The one or more cycles can be repeated at least once, at least twice, at least three, at least four, at least five, at least six times.

[0261] In some aspects, the method further comprises using a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety. An oligonucleotide of a predetermined sequence may be generated by single addition of each pre-determined nucleotide to achieve said sequence.

[0262] In some aspects, the method further comprises using at least two different types of transferases in in separate cycles. Some transferases are better optimized to conjugate or ligate certain modified nucleotides to the oligonucleotide, due to reasons such as steric considerations that influence binding and/or efficient ligation.

[0263] In some aspects, the method comprises elongating the oligonucleotide in a first reaction chamber.

[0264] In some aspects, the method comprises removing the 3′ blocking moiety in a second reaction chamber.

[0265] In some aspects, wherein the first reaction chamber and the second reaction chamber are connected to each other through one or more conduits, and the elongated oligonucleotide comprising the 3′ blocking moiety and the elongated oligonucleotide without the 3′ blocking moiety flows through at least a portion of the one or more conduits. A conduit, such as a conduit that fluidly connects two reaction chambers, may be used to operably link two reaction chambers. In some implementations, the one or more conduits operably links two or more reaction chambers. Non-limiting examples of conduits include chromatography tubing of any material, including glass, plastic, rubber, and silicone. In some aspects, the conduit may be rigid, semi-rigid, or flexible. In some implementations, a conduit may be assembled by connecting two or more conduits. Conduits may be connected using connectors, valves, or fittings. In some aspects, a conduit may be monolithic (e.g., a single, integrated piece). In some aspects, the one or more conduits comprises a set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber. In some aspects, the conduit is capable of bi-directional flow. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber.

[0266] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more pumps. A pump is a solvent delivery system. A pump can deliver a single solvent or multiple solvents. In some implementations, the pump comprises a mixer that combines multiple solvents into a final solvent that

can be delivered to the system. Pumps may be configured to deliver the solvents at a determined flow rate. In some aspects, the one or more pumps deliver solvent at a constant flow rate. In some implementations, the one or more pumps are configured to control a flow rate of the liquid phase. In some aspects, the flow is controlled by a synthesizer.

[0267] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more valves. Valves are used for multiple purposes in a liquid delivery system. Non-limiting examples of valves that can be used in the method include injection or inlet valves that are used to introduce liquid sample into the system, flow path switching valves that may be used to change the flow path of the liquid, column valves for attachment of one or more columns to the system, and outlet valves for waste or sample collection. In some aspects, the one or more valves are multiposition rotary valves. In some aspects, the valves are single position valves. In some aspects, the method further comprises the use of one or more valves that selectively controls a flow pathway of the solution in the system.

[0268] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber by flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the first reaction chamber.

[0269] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber comprising flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the second reaction chamber.

[0270] In some aspects, the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the second reaction chamber.

[0271] In some aspects, the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the first reaction chamber.

[0272] In some aspects, the flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into the first reaction chamber comprises: flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into one or more reservoirs, and flowing the elongated oligonucleotide without the 3′ blocking moiety from the one or more reservoirs into the first reaction chamber.

[0273] Provided herein are methods of template-free synthesis of an oligonucleotide, comprising, in a plurality of reaction chambers : in at least a first reaction chamber, elongating an oligonucleotide in solution by attaching a first nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a

first transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety; flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution from the at least the first reaction chamber into at least a second reaction chamber, wherein the first transferase is retained in the at least the first reaction chamber; in the at least the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed; flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the at least the second reaction chamber into at least a third reaction chamber comprising a second transferase; and in the at least the third reaction chamber, elongating the elongated oligonucleotide without the 3′ blocking moiety the solution by attaching a second nucleotide comprising a 3′ blocking moiety to the elongated oligonucleotide without the 3′ blocking moiety using the second transferase to make a further elongated oligonucleotide comprising the 3′ blocking moiety of the second nucleotide.

[0274] In some aspects, the first transferase and the second transferase are different types of transferases.

[0275] In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide. In some aspects, the first and second nucleotide are different pre-selected types of nucleotides. In some aspects, first nucleotide and the second nucleotide are different types of nucleotides.

[0276] In some aspects, the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase, such as a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase.

[0277] In some aspects, the method further comprises separating the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase. Separating (e.g., substantially separating) can be done by immobilizing the hydrolase on a solid support, or using a filter or membrane to entrap or prevent passage of the hydrolase while the oligonucleotide flows through the pores. In some aspects, the filter or membrane has a MWCO of a pre-determined value.

[0278] In some aspects, the method further comprises repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety. The one or more cycles can be repeated at least once, at least twice, at least three, at least four, at least five, at least six times.

[0279] In some aspects, the method further comprises using a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety. An oligonucleotide of a

predetermined sequence may be generated by single addition of each pre-determined nucleotide to achieve said sequence.

[0280] In some aspects, the method further comprises using at least two different types of transferases in in separate cycles. Some transferases are better optimized to conjugate or ligate certain modified nucleotides to the oligonucleotide, due to reasons such as steric considerations that influence binding and/or efficient ligation.

[0281] In some aspects, the method comprises elongating the oligonucleotide in a first reaction chamber.

[0282] In some aspects, the method comprises removing the 3′ blocking moiety in a second reaction chamber.

[0283] In some aspects, the hydrolase is retained in the at least the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows from the second reaction chamber into the at least the third reaction chamber.

[0284] In some aspects, the first reaction chamber, the second reaction chamber, and the third reaction chamber are connected through a plurality of conduits, wherein: the elongated oligonucleotide comprising the 3′ blocking moiety flows from the at least the first reaction chamber into at least a second reaction chamber through a first portion of the plurality of conduits; and the elongated oligonucleotide without the 3′ blocking moiety flows from the at least the second reaction chamber into at least a third reaction chamber through a second portion of the plurality of conduits. A conduit, such as a conduit that fluidly connects two reaction chambers, may be used to operably link two reaction chambers. In some implementations, the one or more conduits operably links two or more reaction chambers. Non-limiting examples of conduits include chromatography tubing of any material, including glass, plastic, rubber, and silicone. In some aspects, the conduit may be rigid, semi-rigid, or flexible. In some implementations, a conduit may be assembled by connecting two or more conduits. Conduits may be connected using connectors, valves, or fittings. In some aspects, a conduit may be monolithic (e.g., a single, integrated piece). In some aspects, the one or more conduits comprises a set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber. In some aspects, the conduit is capable of bi-directional flow. In some aspects, the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber.

[0285] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more pumps. A pump is a solvent delivery system. A pump can deliver a single

solvent or multiple solvents. In some implementations, the pump comprises a mixer that combines multiple solvents into a final solvent that can be delivered to the system. Pumps may be configured to deliver the solvents at a determined flow rate. In some aspects, the one or more pumps deliver solvent at a constant flow rate. In some implementations, the one or more pumps are configured to control a flow rate of the liquid phase. A synthesizer is a computer interface coupled with a flow system to automate synthesis, such as synthesis of an oligonucleotide. In some aspects, the flow is controlled by a synthesizer.

[0286] In some aspects, the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more valves. Valves are used for multiple purposes in a liquid delivery system. Non-limiting examples of valves that can be used in the method include injection or inlet valves that are used to introduce liquid sample into the system, flow path switching valves that may be used to change the flow path of the liquid, column valves for attachment of one or more columns to the system, and outlet valves for waste or sample collection. In some aspects, the one or more valves are multiposition rotary valves. In some aspects, the valves are single position valves. In some aspects, the method further comprises the use of one or more valves that selectively controls a flow pathway of the solution in the system.

[0287] In some aspects of the methods provided herein, the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog. In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety .

[0288] In some aspects of the methods provided herein, the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution. In some aspects, the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution. In some aspects, the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

[0289] In some aspects of the methods provided herein, the hydrolase is immobilized on a solid support . In some aspects, the hydrolase is immobilized using a covalent, electrostatic, or ionic bond.

[0290] In some aspects of the methods provided herein, the transferase is a polymerase from the DNA polymerase X family. In some aspects, the transferase is a template independent transferase. In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT). In some aspects, the transferase is immobilized on a solid support. In some aspects, the transferase is immobilized using a covalent, electrostatic, or ionic bond.

[0291] In some aspects of the methods provided herein, elongating produces an inorganic pyrophosphate byproduct. In some aspects, the method comprises comprising degrading the inorganic

pyrophosphate using a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the pyrophosphatase is immobilized on a solid support. In some aspects, the pyrophosphatase is immobilized using a covalent, electrostatic, or ionic bond.

[0292] In some aspects of the methods provided herein, the elongating and the degrading occur within the same reaction chamber.

[0293] In some aspects of the methods provided herein the transferase and the inorganic pyrophosphatase are fused together.

[0294] In some aspects of the methods provided herein the transferase and the pyrophosphatase are immobilized on the same solid support.

[0295] In some aspects of the methods provided herein, the transferase and the pyrophosphatase are immobilized on different solid supports.

[0296] In some aspects of the methods provided herein, the transferase is immobilized on a solid support and the pyrophosphatase is in the solution.

[0297] In some aspects of the methods provided herein, the pyrophosphatase is retained in the reaction chamber comprising the transferase, wherein the reaction chamber comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide.

[0298] In some aspects of the methods provided herein, the elongating and the degrading occur within different reaction chambers. In some aspects of the methods provided herein, the methods comprise flowing the elongated oligonucleotide comprising the 3′ blocking moiety and the inorganic pyrophosphate byproduct, in the solution, from a reaction chamber comprising the transferase into a reaction chamber comprising the pyrophosphatase, followed by flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reaction chamber comprising the pyrophosphatase into a reaction chamber in which the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety.

[0299] In some aspects of the methods provided herein, the first reaction chamber or the second reaction chamber is a column.

[0300] In some aspects of the methods provided herein, the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support.

[0301] In some aspects of the methods provided herein, the first reaction chamber comprises a fluidized bed comprising the transferase immobilized on a solid support.

[0302] In some aspects of the methods provided herein, the first reaction chamber comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide.

[0303] In some aspects of the methods provided herein, the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support.

[0304] In some aspects of the methods provided herein, the second reaction chamber comprises a fluidized bed comprising the hydrolase immobilized on a solid support.

[0305] In some aspects of the methods provided herein, the second reaction chamber comprises a filter that prevents passage of hydrolase and allows passage of the oligonucleotide.

[0306] In some aspects of the methods provided herein, the first reaction chamber or the second reaction chamber is a batch reaction chamber.

[0307] In some aspects of the methods provided herein, the batch reaction chamber comprises an impeller.

[0308] In some aspects of the methods provided herein, the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support.

[0309] In some aspects of the methods provided herein, the second reaction chamber comprises a rotating bed reactor comprising hydrolase immobilized on a solid support.

[0310] In some aspects of the methods provided herein, the methods further comprise separating unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide.

[0311] In some aspects of the methods provided herein, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography. In some aspects, the liquid chromatography comprises size exclusion chromatography. In some aspects, the liquid chromatography comprises reverse phase chromatography. In some aspects, the liquid chromatography comprises ion exchange chromatography.

[0312] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis.

[0313] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using tangential flow filtration.

[0314] In further aspects of the methods described herein, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides.

[0315] In further aspects of the methods described herein, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides.

[0316] In further aspects of the methods described herein, the 3′ blocking moiety is a phosphate moiety.

[0317] In further aspects of the methods described herein, the nucleotide comprising the 3′ blocking moiety is a ribonucleotide.

[0318] In some aspects, the nucleotide comprising the 3′ blocking moiety is a deoxyribonucleotide.

[0319] In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification. In some aspects, the 2′ modification is 2′-F or 2′-OMe.

[0320] In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate.

[0321] In further aspects of the methods described herein, the oligonucleotide comprises a 5′ modification.

[0322] Provided herein are methods of template-free synthesis of an oligonucleotide, comprising: elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase in the solution to make an elongated oligonucleotide comprising the 3′ blocking moiety; separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase; removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed.

[0323] In some aspects, separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase comprises flowing the solution through a column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety is a liquid chromatography column. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column. In some aspects, the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide comprising the 3′ blocking moiety. In some aspects, the method further comprises flowing the oligonucleotide and the transferase, in the solution, from a reagent reservoir to the column that retains the transferase, and flowing the oligonucleotide comprising the 3′ blocking moiety, in the solution, from the column that retains the transferase to the reagent reservoir. In some aspects, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety comprises adding a hydrolase to the solution after separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase. In some aspects, the method further comprises separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety from the

hydrolase comprises flowing the solution through a column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column. In some aspects, the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety comprises a filter that prevents passage of the hydrolase and allows passage of the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the method comprises flowing the elongated oligonucleotide without the 3′ blocking moiety and the hydrolase, in the solution, from a reagent reservoir to the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety, and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety to the reagent reservoir.

[0324] In some aspects, the hydrolase is a phosphatase. In some aspects, the hydrolase is an alkaline phosphatase. In some aspects, the method comprises replacing or cleaning the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety or the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the cleaning comprises flowing a buffer through the column to elute the transferase or the hydrolase. In some aspects, the method comprises separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety comprises flowing the solution through a column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a size exclusion column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a reverse phase column. In some aspects, the column that separates the unreacted nucleotides or reaction byproducts from the

elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is an ion exchange column.

[0325] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety using dialysis.

[0326] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety using tangential flow filtration.

[0327] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety after elongating the oligonucleotide by four or more nucleotides.

[0328] In some aspects, the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide only after elongating the oligonucleotide by four or more nucleotides.

[0329] In some aspects, the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

[0330] In some aspects, the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog.

[0331] In some aspects, the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety.

[0332] In some aspects, the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution.

[0333] In some aspects, the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

[0334] In some aspects, the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

[0335] In some aspects, the transferase is a polymerase from the DNA polymerase X family.

[0336] In some aspects, the transferase is a template independent transferase.

[0337] In some aspects, the transferase is a terminal deoxynucleotidyl transferase (TdT).

[0338] In some aspects, the elongating produces an inorganic pyrophosphate byproduct.

[0339] In some aspects, the method further comprises degrading the inorganic pyrophosphate using a pyrophosphatase. In some aspects, the pyrophosphatase is an inorganic pyrophosphatase. In some aspects, the transferase and the inorganic pyrophosphatase are fused together.

[0340] In further aspects of the method, wherein the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety further separates the pyrophosphatase from the elongated oligonucleotide comprising the 3′ blocking moiety.

[0341] In some aspects, the 3′ blocking moiety is a phosphate moiety.

[0342] In some aspects, the nucleotide comprising the 3′ blocking moiety is a ribonucleotide.

[0343] In some aspects, the nucleotide comprising the 3′ blocking moiety is a deoxyribonucleotide.

[0344] In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification. In some aspects, the 2′ modification is 2′-F or 2′-OMe.

[0345] In some aspects, the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate.

[0346] In some aspects, the oligonucleotide comprises a 5′ modification.

[0347] In some aspects, the oligonucleotide is elongated under an inert atmosphere. In some aspects, the oligonucleotide is deblocked under an inert atmosphere. In some aspects, the inert atmosphere is maintained by an inert gas.

[0348] In some aspects, elongating the oligonucleotide is performed between 35 °C and 45 °C. In some aspects, removing the 3′ blocking moiety is performed between 45 °C and 55 °C.

[0349] In some aspects, the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber. In some aspects, the desalting chamber is maintained between 20 °C and 30 °C.

EXAMPLES

[0350] 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.

Example 1: Shake Flask Expression and Purification of Enzymes

Shake Flask Expression

[0351] Selected cultures were plated onto LB agar plates with 1% glucose and 30 μg/ml chloramphenicol and grown overnight at 37 °C. A single colony from each culture was transferred to 5 ml of LB broth with 1% glucose and 30 μg/ml chloramphenicol. The cultures were grown for 20 h at 30 °C, 250 rpm, and subcultured at a dilution of approximately 1:50 into 250 ml of Terrific Broth with 30 μg/ml of chloramphenicol, to a final OD_{600} of about 0.05. The cultures were incubated for approximately 195 min at 30 °C, 250 rpm, to an OD_{600} of about 0.6, and then induced with the addition of IPTG at a final concentration of 1 mM. The induced cultures were incubated for 20 h at 30 °C, 250 rpm. Following this incubation period, the cultures were centrifuged at 4000 rpm x 10 min.

The culture supernatant was discarded, and the pellets were resuspended in 35 ml of 20 mM triethanolamine, pH 7.5. This cell suspension was chilled in an ice bath and lysed using a Microfluidizer cell disruptor (Microfluidics M-110L). The crude lysate was pelleted by centrifugation (11,000 rpm for 60 min at 4 °C), and the supernatant was then filtered through a 0.2 µm PES membrane to further clarify the lysate.

Purification of Enzymes from Shake Flask Lysates

[0352] The clarified lysates were then purified using an AKTA Start purification system and a 5 mL HisTrap FF column (GE Healthcare) using the AC Step HiF setting (the run parameters are provided below in **Table 1**). The SF wash buffer comprised 50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 0.02% v/v Triton X-100 reagent.

Table 1 Purification Parameters

Parameter	Volume
Column volume	5 mL
Flow rate	8 mL/min
Pressure limit	0.3 MPa
Sample volume	35 mL
Equilibration volume	5 column volumes (CV) = 25 mL
Wash Unbound volume	20 CV = 100 mLs
Elution	Isocratic (step)
Elution volume	5 CV = 25 mLs
Fraction volume	1.5 mLs
RE-equilibration volume	5 CV = 25 mLs

[0353] Elution fractions containing protein were identified by UV absorption (A280) and pooled, then dialyzed overnight in dialysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, and 50% glycerol) in a 3.5K Slide-A-Lyzer™ dialysis cassette (Thermo Fisher) for buffer exchange. iPP concentrations from the preparations were measured by absorption at 280 nm.

Example 2: Sample preparation for analysis by capillary electrophoresis (CE) analysis of oligonucleotide reaction products

[0354] For analysis of the reaction samples, capillary electrophoresis was performed using an ABI 3500xl Genetic Analyzer (ThermoFisher). Reactions (1 uL) were quenched by the addition of 99 µL of 1 mM aqueous EDTA. Quenched reactions were diluted in water to 1.25 nM oligonucleotide, and 2 µL of this solution was transferred to a new 96-well MicroAmp Optical PCR plate or 384-well MicroAmp Optical PCR plate containing 18 µL Hi-Di™ Formamide (ThermoFisher) containing an appropriate size standard (LIZ or Alexa633). The ABI3500xl was configured with POP6 polymer, 50 cm capillaries and a 55 °C oven temperature. Pre-run settings were 18KV for 50 sec. Injection was 10KV for 2 sec, and the run settings were 19 KV for 620 sec. FAM-labeled oligo substrates and products were identified by their sizes relative to the sizing ladder.

Example 3: High performance liquid chromatography (HPLC) analysis of nucleotides

[0355] The 5 to 20 µL crude reaction product is diluted to a final total nucleotide concentration of 100 µM nucleotide using 1 mM EDTA in RO water as diluent. 100 µL is transferred from the supernatant to a 96 well round bottom plate and then 10 µL is injected onto an Ultimate 3000 HPLC system using a PAL autosampler according to the method outlined in **Table 3.1**.

Table 3.1. Achiral HPLC Parameters (Ion Pairing Gradient)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Agilent Zorbax RR StableBond Aq, 150 x 3.0 mm x 3.5 µm
Guard Column	Agilent Zorbax StabeBond Aq, 5.0 x 3.0 mm, 1.8 µm
Mobile Phases	A: 50 mM sodium phosphate, 2 mM tetrabutylammonium bisulfate, pH 7.0 B: Acetonitrile C: Water
LC Gradient	- 0 – 4.0 min: 9.0 % B, 48.5 – 16.2 % C - 4.0 – 4.5 min: 9.0 % B, 16.2 – 48.5 % C - 4.5 – 5.5 min: 9.0 % B, 48.5 % C
Flow rate	1.2 mL/min
Run time	5.5 min
Column temperature	30 °C
Injection volume	10 µL
UV Detector	254 nm

Example 4: High performance liquid chromatography (HPLC) analysis of oligonucleotides

[0356] A 2 to 10 µL aliquot is removed from the reaction and diluted to a concentration of 25 µM oligonucleotide using 1 mM EDTA (pH 8) in RO water as diluent. 10 µL from the diluted samples is then injected onto an Ultimate 3000 HPLC system using a PAL autosampler according to either Method A outlined in **Table 4.1**, or Method B outlined in **Table 4.2**.

Table 4.1. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method A)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Agilent Advance Bio Amino Acid Analysis (AAA), 3.6x100 mm, 2.7 µm
Mobile Phases	A: 100 mM triethylamine acetate, pH 7.0 B: Acetonitrile
LC Gradient	- 0 – 1.0 min: 8.0% B - 1.0 – 8.0 min: 8.0 – 14.0% B - 8.0 – 9.0 min: 8.0% B
Flow rate	- 1.0 mL/min
Run time	9.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Table 4.2. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method B)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler

Table 4.2. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method B)	
Column	Agilent Advance Bio Amino Acid Analysis (AAA), 3.6x100 mm, 2.7 µm
Mobile Phases	A: 100 mM triethylamine acetate, pH 7.0 B: Acetonitrile
LC Gradient	- 0 – 1.0 min: 11.0% B - 1.0 – 5.0 min: 11.0 – 13.0% B - 5.0 – 7.0 min: 11.0% B
Flow rate	- 0.7 mL/min
Run time	7.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Example 5: Oligonucleotide Analysis by Liquid Chromatography Mass Spectrometry (LCMS)

[0357] Samples were pre-diluted to between 25 and 75 µM oligonucleotide in RO water and injected on an Agilent 6470B triple quadrupole LCMS system according to the method outlined in **Table 5.1** below.

Table 5.1. Oligonucleotide Analysis by LCMS	
Instrument	Agilent 6470B
Column	Waters XTerra MS C18 5 µm, 2.1 x 50 mm, with Phenomenex Guard
Mobile Phases	B: 15 mM dibutylamine and 25 mM hexafluoroisopropanol in water C: Methanol
LC Gradient	- 0 – 0.5 min: 10% B, 90% C - 0.5 - 10 min: 45% B, 55% C - 10 – 11.5 min: 45 – 75% B, 55 – 20% C - 11.5 – 11.9 min: 75% B, 20% C - 11.9 – 12 min: 75 – 10% B, 20 – 90% C - 12 – 14 min: 10% B, 90% C
Flow rate	- 0.6 mL/min
Run time	14 min
Column temperature	50 °C
Injection volume	6 µL
UV Detector	260 nm
MS-Mode	Negative
Scan Data	950-2050
Scan Time	400
Frag Voltage	200 V
Drying Gas	12 L/min, 275 °C
Sheath Gas	12 L/min, 350 °C
Nebulizer Pressure	35 psi
Cap Voltage	4000 V
Nozzle Voltage	2000 V

Example 6: Residual Alkaline Phosphatase Assay

[0358] Residual alkaline phosphatase (AP) is measured by reaction with *p*-nitrophenyl phosphate. Using a concentrated stock of the desired alkaline phosphatase variant to be measured, a 100 µL diluted sample containing 2.0 µM enzyme is prepared, using the same buffer as the analyte as diluent. 20 µL from the 2.0 µM stock is serially diluted by 2x across one row of a biorad 96-well PCR plate using analyte buffer as diluent, with a final volume of 10 µL in each well (final concentrations ranging from 2.0 to 0.001 µM enzyme). Then, 10 µL is removed from each sample to be analyzed and transferred to one well of the biorad 96-well PCR plate.

[0359] A reaction buffer stock is then prepared containing: *p*-nitrophenylphosphate (2.0 mM), $CoCl_2$ (1.0 mM), TEoAHCl (50 mM, pH 7.8). 10 µL is transferred from the reaction stock to the wells containing analyte and serially diluted AP on the biorad 96-well PCR plate. The plate is heat sealed with an aluminum seal, mixed, and briefly centrifuged to collect the liquid in the bottom of each well. The plate is then heated at 50 °C for 10 minutes on a thermal cycler. Afterward, the reaction is quenched by diluting each well with 100 µL of 1.0 M NaOH. The samples are transferred to a clear flat-bottom 96-well plate for analysis by UV-Vis absorbance at 405 nm on a SpectraMax M2 plate reader. A linear least-squares fitting to the linear portion of the AP dose response curve from 0.0005 µM to 0.05 µM is used to measure the amount of AP in the analyte wells.

Example 7: Residual $CoCl_2$ Assay

[0360] A 10 mM $CoCl_2$ solution was prepared in the same buffer as the analyte to be assayed and serially diluted across 12 wells of a clear flat bottom plate so that each well had 50 µL and the last well was blank. A 100 µL aliquot was removed from each sample to be measured and diluted 2x across three wells in the same clear bottom plate so that each sample well contained 50 µL.

[0361] A working reagent stock was prepared using solutions A, B, and C from a Micro BCA™ protein assay kit purchased from Thermo Scientific according to the directions provided.

[0362] An equal volume of 50 µL was added from the BCA working reagent to each well in the clear bottom plate and mixed using a pipette. The plate was sealed and allowed to incubate at room temperature for 15 to 30 minutes before analyzing the absorbance at 562 nm on a SpectraMax M2 plate reader.

[0363] The absorbances of the serially diluted wells from the known 10 mM CoCl2 stock used as a calibration curve, with the linear portion of the curve fit with a linear least squares fitting. The slope was then used to determine the concentration of $CoCl_2$ in each sample, accounting for dilution and averaged.

Example 8: Enzyme Immobilization Procedure on Affinity Resins

[0364] The TnT or AP was first produced in shake flask and purified as described in Example 1. The storage buffer from an aliquot of the desired protein stock was then exchanged for TEAHCl (500 mM, pH 7.8) by diluting 10x with TEAHCl followed by concentration through a Sartirous VivaSpin 6 (30,000 MWCO) spin filter at 4000 rpm and 4 °C. The resulting small volume is then diluted another 2.5 x with TEAHCl (500 mM, pH 7.8) to give the original volume.

[0365] Between 2 and 50 mg affinity resin was weighed out, either wet or dry, into a 96 well plate with a volume of 2 mL per well or into a 2 mL Eppendorf tube. For larger preparations, 0.5 to 1.0 g adsorbent or ion-exchange resin was weighed out into a 15 mL conical tube. The volume of protein stock containing the desired wt. % of protein vs resin mass was transferred to the 96 well plate or tube containing resin.

[0366] The samples were then incubated at 4-25 °C for 24 to 48 h using either 500 rpm agitation or on a tube rotator operating at 10 rpm. Before subjecting to activity assays, the supernatant was removed, and the resin was washed three times. For smaller preparations utilizing adsorbent or ion-exchange resins in 96 well plates or 2.0 mL Eppendorf tubes, 300 µL wash volumes of TEAHCl (50 mM, pH 7.8) were used, allowing the mixture to agitate at room temperature at 500 rpm for 5 minutes during each wash. Between each wash the tubes were briefly centrifuged to collect resin at the bottom. In the case of a larger preparation in a 15 mL conical tube, the resin was collected by vacuum filtration and then washed three times with 10 mL of TEAHCl (50 mM, pH 7.8), allowing the resin to mix well on a rotator for 10 minutes at room temperature during each wash.

Example 9: Co-Immobilization of TnT/iPP and Enzymatic Extension of 5'-AAAAmC*mA*mG and 5'-AAAAfGmUfG on Affinity Resin

[0367] TnT enzyme variant (SEQ ID NO: 2) was produced and purified according to Example 1.

[0368] iPP enzyme variant (SEQ ID NO: 8) was produced and purified according to Example 1.

Extension of 5'-AAAAmC*mA*mG with mATP-3'P

[0369] Between 10 to 20 mg of Co(II)-loaded ChiralVision IB-HIS-2 resin was weighed out into 8 Eppendorf tubes. TnT and iPP were co-immobilized on the resin at 2.5 wt% and 0.2 wt% vs wet weight of resin, respectively, according to Example 8. After washing, the resins were transferred to 8 wells of a 1.0 mL deep 96-well plate.

[0370] Eight reaction stocks comprising four oligo concentrations matrixed with two $CoCl_2$ concentrations were prepared, each containing: 5'-AAAAmC*mA*mG (800, 400, 200, or 100 µM), mATP-3'P (1.2 equivalents vs oligo), $CoCl_2$ (1.0 or 0.25 mM), TEoAHCl (50 mM), pH 7.8.

[0371] To the wells containing co-immobilized TnT and iPP was added 10 µL/mg of reaction buffer vs resin wet weight. The resins were incubated with reaction buffer for 1 h at 50 °C and 500 rpm

agitation. A 10 µL aliquot was then removed from each reaction and diluted to 25 µM oligonucleotide for analysis by HPLC according to Example 4, Method B. The conversion of 5'-AAAAmC*mA*mG to 5'-AAAAmC*mA*mGmA-3'P is reported in **Table 9.1**.

Table 9.1: Activity of TnT (SEQ ID NO: 2) and iPP (SEQ ID NO: 8) Co-immobilized on Affinity Resin

5'-AAAAmC*mA*mG (µM)	$CoCl_2$ (mM)	Percent Conversion to 5'-AAAAmC*mA*mGmA-3'P
800	0.25	+
400	0.25	++
200	0.25	++
100	0.25	++
800	1.0	++
400	1.0	++
200	1.0	+
100	1.0	+
Levels of conversion are determined by ratio of AUC Product vs AUC Substrate in HPLC Chromatogram and defined as follows: "+" 40 to 50%, "++" 50 to 60%.		

Extension of 5'-AAAAfGmUfG with mUTP-3'P at 0.2 wt% iPP Loading

[0372] Between 10 to 20 mg of Co(II)-loaded ChiralVision IB-HIS-2 resin was weighed out into 12 Eppendorf tubes. TnT and iPP were co-immobilized on the resin at 2.5 wt% and 0.2 wt% vs wet weight of resin, respectively, according to Example 8. After washing, the resins were transferred to 12 wells of a 1.0 mL deep 96-well plate.

[0373] Twelve reaction stocks comprising four oligonucleotide concentrations matrixed with three $CoCl_2$ concentrations were prepared, each containing: 5'-AAAAfGmUfG (3200, 1600, 800, or 400 µM), mUTP-3'P (2.0 equivalents vs oligo), $CoCl_2$ (2.0, 1.0, or 0.5 mM), TEoAHCl (50 mM), pH 7.8.

[0374] To the wells containing co-immobilized TnT and iPP was added 20 µL/mg of reaction buffer vs resin wet weight. The resins were incubated with reaction buffer for 24 h at 50 °C and 500 rpm agitation. A 10 µL aliquot was then removed from each reaction and diluted to 25 µM oligonucleotide in 1 mM EDTA for analysis by HPLC according to Example 4, Method A. A second 10 µL aliquot was removed and diluted to 100 µM nucleotide using 1 mM EDTA and analyzed by HPLC according to Example 4. The conversion of 5'-AAAAfGmUfG to 5'-AAAAfGmUfGmU-3'P along with the percent unhydrolyzed mUTP-3'P in the reaction stock after 24h is reported in **Table 9.2**. The percent unhydrolyzed mUTP-3'P is calculated as the $\frac{AUC_{mUTP-3'P}}{AUC_{total}}$ taken from the HPLC chromatogram, representative of the percent of total remaining nucleotide that is constituted of unhydrolyzed mUTP-3'P and does not take into account mUTP-3'P consumed in the reaction with TnT and oligonucleotide.

Table 9.2 NTP-3'P Hydrolysis Activity of TnT (SEQ ID NO: 2) and iPP (SEQ ID NO: 8) Co-immobilized on Affinity Resin			
5'-AAAAfGmUfG (μM)	**$CoCl_2$ (mM)**	**Percent Conversion to 5'-AAAAfGmUfGmU-3'P**	**Percent Unhydrolyzed mUTP-3'P**
3200	2.0	++	+++
1600	2.0	++	+++
800	2.0	+	+
400	2.0	+	+
3200	1.0	++	+++
1600	1.0	++	+++
800	1.0	++	+++
400	1.0	+	++
3200	0.5	+	+++
1600	0.5	+	+++
800	0.5	+	+++
400	0.5	+	+++
Percent conversion to 5'-AAAAfGmUfGmU-3'P are determined by ratio of AUC Product vs AUC substrate in the HPLC chromatogram and defined as follows: "+" 5 to 10%, "++" 10 to 20%. Levels of unhydrolyzed NTP-3'P are determined by ratio of AUC Product vs AUC Substrate in HPLC chromatogram and defined as follows: "+" 0 to 30%, "++" 30 to 70%, "+++" 70 to 90%.			

Extension of 5'-AAAAfGmUfG with mUTP-3'P at 0.02 wt% iPP Loading

[0375] Between 10 to 20 mg of Co(II)-loaded ChiralVision IB-HIS-2 resin was weighed out into 12 Eppendorf tubes. TnT and iPP were co-immobilized on the resin at 2.5 wt% and 0.02 wt% vs wet weight of resin, respectively, according to Example 8. After washing, the resins were transferred to 12 wells of a 1.0 mL deep 96-well plate.

[0376] Twelve reaction stocks comprising four oligonucleotide concentrations matrixed with three $CoCl_2$ concentrations were prepared, each containing: 5'-AAAAfGmUfG (3200, 1600, 800, or 400 μM), mUTP-3'P (2.0 equivalents vs oligo), $CoCl_2$ (2.0, 1.0, or 0.5 mM), TEoAHCl (50 mM), pH 7.8.

[0377] To the wells containing co-immobilized TnT and iPP was added 20 μL/mg of reaction buffer vs resin wet weight. The resins were incubated with reaction buffer for 24 h at 50 °C and 500 rpm agitation. A 10 μL aliquot was then removed from each reaction and diluted to 25 μM oligonucleotide in 1 mM EDTA for analysis by HPLC according to Example 4, Method A. A second 10 μL aliquot was removed and diluted to 100 μM nucleotide using 1 mM EDTA and analyzed by HPLC according to Example 3. The conversion of 5'-AAAAfGmUfG to 5'-AAAAfGmUfGmU-3'P along with the percent unhydrolyzed mUTP-3'P in the reaction stock after 24h is reported in **Table 9.3**. The percent unhydrolyzed mUTP-3'P is calculated as the $\frac{AUC_{mUQP-3'P}}{AUC_{total}}$ taken from the HPLC chromatogram, representative of the percent of total remaining nucleotide that is constituted of unhydrolyzed mUTP-3'P and does not take into account mUTP-3'P consumed in the reaction with TnT and oligonucleotide.

Table 9.3 NTP-3'P Hydrolysis Activity of TnT (SEQ ID NO: 2) and iPP (SEQ ID NO: 8) Co-immobilized on Affinity Resin with Low iPP Loading			
5'-AAAAfGmUfG (μM)	**$CoCl_2$ (mM)**	**Percent Conversion to 5'-AAAAfGmUfGmU-3'P**	**Percent Unhydrolyzed mUTP-3'P**
3200	2.0	+	++
1600	2.0	++	++
800	2.0	++	++
400	2.0	++	+
3200	1.0	+	++
1600	1.0	+	++
800	1.0	+	++
400	1.0	++	++
3200	0.5	+	++
1600	0.5	+	++
800	0.5	+	++
400	0.5	+	+
Percent conversion to 5'-AAAAfGmUfGmU-3'P are determined by ratio of AUC Product vs AUC substrate in the HPLC chromatogram and defined as follows: "+" 10 to 20%, "++" 20 to 30%. Levels of conversion are determined by ratio of AUC Product vs AUC Substrate in HPLC Chromatogram and defined as follows: "+" 70 to 90%, "++" 90 to 100%.			

Example 10: Co-Immobilized TnT SEQ ID NO: 2/iPP SEQ ID NO: 8 and TnT SEQ ID NO: 4/iPP SEQ ID NO: 8 Enzymatic Extension of 5'-AAAAfGmUfGmU

[0378] TnT enzyme variant (SEQ ID NO: 2) was produced and purified according to Example 1.

[0379] TnT enzyme variant (SEQ ID NO: 4) was produced and purified according to Example 1.

[0380] iPP enzyme variant (SEQ ID NO: 8) was produced and purified according to Example 1.

[0381] Between 10 to 20 mg of Co(II)-loaded ChiralVision IB-HIS-2 resin was weighed out into 8 Eppendorf tubes. On two resin samples, TnT (SEQ ID NO: 2) and iPP were co-immobilized 2.5 wt% and 0.2 wt% vs wet weight of resin, respectively, according to Example 8. On another two resin samples, TnT (SEQ ID NO: 2) and iPP were co-immobilized 2.5 wt% and 0.02 wt% vs wet weight of resin, respectively, according to Example 8. On another two resin samples, TnT (SEQ ID NO: 4) and iPP were co-immobilized 2.5 wt% and 0.2 wt% vs wet weight of resin, respectively, according to Example 8. On the last two resin samples, TnT (SEQ ID NO: 4) and iPP were co-immobilized 2.5 wt% and 0.02 wt% vs wet weight of resin, respectively, according to Example 8. After washing, the resins were transferred to 8 wells of a 1.0 mL deep 96-well plate.

[0382] Using a previous reaction product containing a 60:40 mix of 5'-AAAAfGmUfG: 5'-AAAAfGmUfGmU, two reaction stocks were prepared using two different $CoCl_2$ concentrations, consisting of: 5'-AAAAfGmUfG (480 μM), 4A1m (320 μM), fCTP-3'P (1600 μM), $CoCl_2$ (0.7 or 1.5 mM), TEoAHCl (50 mM), pH 7.8.

[0383] To the wells containing co-immobilized TnT and iPP was added 10 µL/mg of reaction buffer vs resin wet weight. The resins were incubated with reaction buffer for 20 min at 50 °C and 500 rpm agitation. A 2 µL aliquot was then removed from each reaction and diluted to 25 µM oligonucleotide in 1 mM EDTA for analysis by HPLC according to Example 4, Method A. The conversion of 4A1m to 5'-AAAAfGmUfGmUfC-3'P for each resin preparation is listed in **Table 10.1**.

Table 10.1: Activity of TnT variant (SEQ ID NO: 4) and variant (SEQ ID NO: 2) Co-immobilized with SEQ ID NO: 8 on Affinity Resin

TnT Variant	$CoCl_2$ (mM)	iPP Loading on Resin (Weight Percent vs Wet Weight)	Percent Conversion to 5'-AAAAfGmUfGmUfC-3'P
SEQ ID NO: 2	0.7	0.02	+
SEQ ID NO: 2	1.5	0.02	+
SEQ ID NO: 2	0.7	0.2	+
SEQ ID NO: 2	1.5	0.2	+
SEQ ID NO: 4	0.7	0.02	++
SEQ ID NO: 4	1.5	0.02	++
SEQ ID NO: 4	0.7	0.2	++
SEQ ID NO: 4	1.5	0.2	++
Levels of conversion are determined by ratio of AUC Product vs AUC Substrate in HPLC Chromatogram and defined as follows: "+" 40 to 70%, "++" 80 to 100%.			

Example 11: Immobilization and Activity of Alkaline Phosphatase Variant SEQ ID NO: 10 on Epoxide Resin

Immobilization on Epoxy Resin

[0384] Alkaline phosphatase SEQ ID NO: 10 was produced and purified, as described in Example 1 and as described in patent application No. PCT/US2023/076667, filed October 12, 2023, which is incorporated by reference herein in its entirety.

[0385] To remove storage buffer, the AP was diluted 10x into 20 mM MOPS (pH 7.0) then concentrated through a 10 kDa spin filter. The concentrated stock was diluted further with 20 mM MOPS (pH 7.0) to produce a 0.1 g/L and a 1.0 g/L master stock.

[0386] Epoxide functionalized resins purchased from ChiralVision (IB-COV-2 and IB-COV-7) were weighed out in 10 to 20 mg quantities in 12 Eppendorf tubes to give 6 tubes with IB-COV-2 and 6 tubes with IB-COV-7. Each resin sample was washed one time with 150 µL 20 mM MOPS (pH 7.0). Using the 1.0 g/L and 0.1 g/L AP master stocks, 25 µL stock per mg resin was added to the Eppendorf tubes to give either 2.5 wt.% or 0.25 wt.% AP loading, respectively. The tubes were placed on a rotator set to 3 rpm and incubated at 4 °C for 48 h.

[0387] A 10 µL sample was removed from the supernatant in each tube as well as the two master stock solutions, diluted 100x in MOPS (pH 7.0), and tested for residual AP activity according to Example 6. The difference in activity between the supernatants and master stock were used to

calculate the percent of AP in supernatant that became immobilized. Each percent in **Table 11.1** is the average of three immobilization reactions, presented as the weight percent loading vs wet weight of resin.

Table 11.1. Immobilization of AP SEQ ID NO: 10 on Epoxy Resin		
Resin	**Targeted AP Weight Percent Loading**	**Percent of Target Loading**
IB-COV-2	0.25	++
IB-COV-2	2.5	++
IB-COV-7	0.25	++
IB-COV-7	2.5	+
Levels of immobilization are determined by ratio of A280 vs negative control and defined as follows: "+" 45 to 60, "++" 80 to 100%.		

Phosphorolysis Activity of AP variant SEQ ID NO: 10 on Epoxy Resin

[0388] Each sample was washed with one of three wash methods: (1) Three 150 µL washes of 20 mM MOPS (pH 7.0); (2) 300 µL of 1 M ethanolamine in 20 mM MOPS (pH 7.0) for 4 h at room temperature followed by three 150 µL washes of 20 mM MOPS (pH 7.0); or (3) 300 µL of 50 mM sodium phosphate (NaPi) buffer for 24 h at 4 °C followed by three 150 µL washes of 20 mM MOPS (pH 7.0).

[0389] A reaction stock was prepared consisting of: 5'-(FAM)T_{15}mAmG*mGmA-3'P (0.225 µM), 5'-T_{15}mAmG*mGmA-3'P (199.8 µM), $CoCl_2$ (0.25 mM), TEoAHCl (20 mM), pH 7.8. Using the measured weight percent loading of AP on resin in each sample, a volume of reaction stock was added such that the final on-resin AP was 0.4 or 4.0 mg/mL for resins loaded at a targeted 0.25 or 2.5 weight percent vs wet weight of resin, respectively. The samples were then heated at 50 °C for 15 minutes.

[0390] A 2 µL aliquot was removed from the supernatant of each sample and analyzed by capillary electrophoresis, as described in Example 2.

Table 11.2 Activity of AP SEQ ID NO: 10 on Epoxy Resin			
Resin	**Targeted AP Weight % Loading**	**Resin Wash**	**Percent Dephosphorylated Product: 5'-(FAM)T_{15} mAmG*mGmA**
IB-COV-2	0.25	MOPS	++
IB-COV-2	0.25	Ethanolamine	+
IB-COV-2	0.25	NaPi	++
IB-COV-2	2.5	MOPS	+++
IB-COV-2	2.5	Ethanolamine	++
IB-COV-2	2.5	NaPi	+++
IB-COV-7	0.25	MOPS	+++
IB-COV-7	0.25	Ethanolamine	+
IB-COV-7	0.25	NaPi	++
IB-COV-7	2.5	MOPS	+++
IB-COV-7	2.5	Ethanolamine	++

Table 11.2 Activity of AP SEQ ID NO: 10 on Epoxy Resin			
IB-COV-7	2.5	NaPi	+++
Levels of conversion are determined by ratio of AUC Product vs AUC Substrate in HPLC Chromatogram and defined as follows: "+" 0 to 30%, "++" 30 to 70%, "+++" 70 to 100%.			

Example 12: General Procedure for the Post-Immobilization Treatment of Purified Alkaline Phosphatase on Covalent Resin

[0391] Alkaline phosphatase (AP) enzyme SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0392] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 11.

[0393] After immobilization was complete, a 20-100 mg portion of epoxide functionalized resin loaded with 2.0 wt. % AP was weighed out into a 2 mL Eppendorf tube. A 50-300 mM aliquot of quench reagent dissolved in TEAHCl (50 mM) was added to each tube and the tubes shook at 600 rpm for 0.5-3 h at 25-60 °C. After incubation, the quench solution was removed, and the resin washed with three portions of TEAHCl (50 mM, pH 7.8). The wash solution was discarded, and the quenched resins stored at 4 °C until ready for use.

Example 13: Post-Immobilization Treatment of Purified Alkaline Phosphatase on Covalent Resin and Enzymatic 3'-Dephosphorylation of RNA Oligomer

[0394] Alkaline phosphatase (AP) enzyme variant (SEQ ID NO: 12) was produced in shake flask and purified as described in Example 1.

[0395] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 11.

[0396] The AP loaded epoxide functionalized resins were quenched according to the procedure described in Example 12.

[0397] After post-immobilization treatment of the epoxide functionalized resins loaded with 2.0 wt. % AP was complete, a 30-50 mg portion of resin was weighed out into 2 mL Eppendorf tubes.

[0398] A reaction solution comprised of 800 μM 5'-AAAAmC*mA*mGmA-3'P, residual mATP-3'P, 1.0 mM $CoCl_2$, and 220 μM iPP in TEAHCl (50 mM, pH 7.8) was prepared. A 300-500 μL portion of this reaction solution was added to each of the 2 mL Eppendorf tubes containing epoxide functionalized resins loaded with 2.0 wt. % AP treated with a post-immobilization quench reagent. The reaction was then shaken at 45 °C, 600 rpm for 1 h. After the reaction was complete, a 5 μL aliquot of the reaction was diluted to 160 μL in 1 mM EDTA and analyzed via the HPLC method as described in Example 4, Method B.

Table 13.1. 3'P-Dephosphorylation Using AP Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Treatment		
Reagent	**Concentration**	**Percent 5'-AAAA-mC*mA*mGmA**
Sodium Thiosulfate	60 mM	+
L-cysteine	200 mM	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" >95%.		

Example 14: Extension and Deblocking Reactions in Spin Reactors

[0399] TnT enzyme variant (SEQ ID NO: 4) was produced and purified according to Example 1.

[0400] iPP enzyme variant (SEQ ID NO: 8) was produced and purified according to Example 1.

[0401] TnT and iPP were immobilized on ChiralVision IB-HIS-2 resin according to Example 8.

Extension of 5'-AAAAmC*mA*mG with mATP-3'P

[0402] Between 200 and 300 mg of Co(II)-loaded IB-HIS-2 resin already charged with 2.5 wt. % TnT and 0.02 wt. % iPP was weighed out into a ChemSpin MagRBR spin reactor. This spin reactor with resin was loaded into a plastic tube provided with the spin reactor.

[0403] A reaction stock (4 mL) was prepared containing: 5'-AAAAmC*mA*mG (1 mM), mATP-3'P (1.5 mM) $CoCl_2$ (1.0 mM), TEAHCl (50 mM), pH 7.8.

[0404] This reaction master mix was added to the plastic tube containing the resin loaded spin reactor. The reaction was then incubated at 50 °C for 4 h with magnetic stirring at 400 rpm.

[0405] After 0, 1, 2, 3, and 4 h, 3.75 µL aliquots were removed from the reaction and diluted to 25 µM oligonucleotide in 1 mM EDTA for analysis by HPLC according to Example 4, Method B. The conversion of 5'-AAAAmC*mA*mG to 5'-AAAAmC*mA*mGmA-3'P is reported in **Table 14.1**.

Table 14.1 Activity of TnT variant (SEQ ID NO: 4) and iPP variant (SEQ ID NO: 8) Co-immobilized on Affinity Resin in a Spin Reactor	
Time (h)	**Percent Conversion to 5'-AAAAmC*mA*mGmA-3'P**
0	-
1	+
2	++
3	++
4	++
Levels of conversion are determined by ratio of AUC Product vs AUC Substrate in HPLC Chromatogram and defined as follows: "-" 0%, "+" 70 to 90%, "++" >90%.	

AP Catalyzed 3'-Dephosphorylation of RNA Oligomer (5'-AAAAmC*mA*mGmA-3'P)

[0406] ChemSpin MagRBR spin reactors loaded with 250 mg IB-COV-7 resin, charged with 2 and 1 wt. % AP were loaded into two of the plastic tubes containing the reaction mixture resulting from the

extension reaction described above. AP was added to the remaining plastic tube such that the same amount of enzyme was added in the tube as was immobilized on the 2 wt% sample. The reaction was then incubated at 50 °C for 2 h at 400 rpm.

[0407] After 1.25, and 2 h, 15 µL aliquots of each of the supernatants was transferred into an Eppendorf tube containing 135 µL of 1 mM EDTA for HPLC analysis according to Example 4, Method B.

Table 14.2 Dephosphorylation of mATP-3'P Catalyzed by Immobilized and Free AP in Spin Reactors

AP	Conversion (%)
Immobilized – 2 wt%	+
Immobilized – 1 wt%	+
Free – 2 wt% equivalent	++
Conversion of nucleotide was determined as percent disappearance of starting oligonucleotide in the HPLC trace and defined as follows: "+" 80 to 90%, "++" > 90%	

Example 15: General Procedure for Enzymatic RNA Oligomer Extensions Using Co-immobilized TnT and iPP in a Multi-Pass Flow Reactor

[0408] TnT variant (SEQ ID NO: 4) and IPP variant (SEQ ID NO: 8) were co-immobilized on ChiralVision IB-HIS-2 resin, pre-loaded with $CoCl_2$, at 2.5 and 0.02 wt. % vs wet weight of resin, respectively, according to Example 8. Additional preparations of TnT and iPP co-immobilized on ChiralVision IB-HIS-2 resin at 2.5 and 0.2 wt. % were prepared according to Example 8 as needed.

[0409] A BioRad Econo Alpha column (manufacturer part # 12009463) with internal dimensions of 6.6 mm x 50 mm (650 µL internal volume), fitted with an adjustable flow adapter containing 5 um pore size frits, was packed with 450 mg of TnT and iPP co-immobilized on ChiralVision IB-HIS-2 resin at 2.5 and 0.02 wt. %, respectively; a small portion of TEAHCl (50 mM, pH 7.8) was used to aid in the transfer of resin to the column. The flow adapter was used to compress the resin to give a bed volume of approximately 500 µL. The column was fitted with a resistive heating jacket to maintain a bed temperature of 40-60 °C.

[0410] A 15 mL Falcon tube was charged with 50 mM TEAHCl buffer at pH 7.8, 100-2000 µM RNA oligomer, 0.5-2.0 mM $CoCl_2$, and 100-4000 µM NTP-3'P; the reaction reservoir was stored at 4-25 °C for the duration of the flow reaction. This solution was recirculated via peristaltic pump at a flow rate of 0.04-0.50 mL/min through the temperature-controlled column containing TnT and iPP co-immobilized on IB-HIS-2 at 2.0 and 0.02 wt%, respectively. The endpoint of the reaction was determined by analyzing an aliquot removed from the reservoir via HPLC, as described in Example 4.

[0411] Upon completion of the reaction, the reaction solution was flushed from the system via peristaltic pump and the column washed with TEAHCl (50 mM, pH 7.8) to collect retained RNA

oligomer. Aliquots of the wash buffer were analyzed by HPLC for the presence of RNA oligomer as described in Example 4.

Example 16: Procedure for Enzymatic 3-Dephosphorylation of an RNA Oligomer Using Immobilized Alkaline Phosphatase in a Single-Pass Flow Reactor

[0412] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 11. Additional preparations of alkaline phosphatase immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % were prepared according to Example 11 as needed.

[0413] A BioRad Econo Alpha column (manufacturer part # 12009463) with internal dimensions of 6.6 mm x 50 mm (650 µL internal volume), fitted with an adjustable flow adapter containing 5 um pore size frits, was packed with 450 mg of alkaline phosphatase immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. %; a small portion of TEAHCl (50 mM, pH 7.8) was used to aid in the transfer of resin to the column. The flow adapter was used to compress the resin to give a bed volume of approximately 500 µL. The column was fitted with a resistive heating jacket to maintain a bed temperature of 40-60 °C.

[0414] A 15 mL Falcon tube containing the crude extension reaction mix, comprised of 50 mM TEAHCl buffer at pH 7.8, 100-1600 µM RNA oligomer, 0.5-2.0 mM $CoCl_2$, and 200-3200 µM NTP-3'P, was pumped via peristaltic pump at a flow rate of 0.04-0.50 mL/min through the temperature-controlled column containing alkaline phosphatase immobilized at 2.0 wt. % on ChiralVision IB-COV-7. After passing through the column containing immobilized alkaline phosphatase, the solution was collected in a separate collection vessel. The endpoint of the reaction was determined by analyzing an aliquot removed from the reservoir via HPLC, as described in Example 4. Residual NTP-3'P dephosphorylation was analyzed by the HPLC method as described in Example 3.

Example 17: Leaching of Alkaline Phosphatase Immobilized on Epoxy Resin in a Single Pass Flow Reactor

[0415] Alkaline phosphatases (Aps) SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 were immobilized onto IB-COV-7 according to Example 11 and packed into a BioRad Econo Alpha column according to Example 16.

[0416] The loaded resin was washed with 50 mM TEAHCl (pH 7.8) at 0.7 ml/min. Wash fractions were collected in volume of 420 µl and the residual AP activity assay was performed as in the Example 6. The percent enzyme detected in wash fractions was calculated as ratio between enzyme detected in wash fraction and total bound enzyme and multiplied with 100 shown in **Table 17.1**.

Table 17.1 In-flow Leaching of AP Immobilized on Epoxy Resin

SEQ ID NO:	Percent Enzyme Detected in Wash Fractions
10	+

Table 17.1 In-flow Leaching of AP Immobilized on Epoxy Resin	
12	++
14	++
Levels of decreased enzyme leaching were determined relative to the reference polypeptide of SEQ ID NO: 10 and defined as follows: "+" > 20%, "++" <20%.	

Example 18: Activity of Alkaline Phosphatase Immobilized on Epoxy Resin in a Single Pass Flow Reactor

[0417] Immobilized alkaline phosphatases (Aps) SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 were assayed for activity with mATP-3'P and species decomposition was analyzed by HPLC (see Example 11).

[0418] Reactions were performed in flow using the immobilized enzyme from Example 17. 10 mL of solution containing 1 mM mATP-3'P, 50 mM triethanolamine (pH 7.8), and 500 μM $CoCl_2$ was ran through the column at the rate of 0.07 mL/min for 2 hours at 50 °C. 1 mL fractions were collected and the HPLC analysis from Example 3 was performed.

[0419] Activity relative to SEQ ID NO: 10 (Activity FIOP) was calculated as the percent mATP-3'P of the variant compared with the percent mATP-3'P observed by the reaction with SEQ ID NO: 10. The results are shown in **Table 18.1**.

Table 18.1 Activity of AP Immobilized on Epoxy Resin in a Single Pass	
SEQ ID NO:	**FIOP mATP-3'P Peak Area Relative to SEQ ID NO: 10**
SEQ ID NO: 12	++
SEQ ID NO: 14	+
Levels of increased activity were determined relative to the reference polypeptide of SEQ ID NO: 10 and defined as follows: "+" >1.0, "++" <1.0.	

Example 19: Iterative Extension of an RNA Oligomer in a Multi-Pass Flow Reactor

[0420] TnT enzyme variant (SEQ ID NO: 4) was produced in shake flask and purified as described in Example 1.

[0421] Inorganic pyrophosphatase (iPP) variant (SEQ ID NO: 8) as produced and purified, as described in Example 1.

[0422] Alkaline phosphatase (AP) variant (SEQ ID NO: 10) was produced and purified, as described in Example 1.

[0423] TnT and iPP were co-immobilized on ChiralVision IB-HIS-2 resin (3 g), pre-loaded with $CoCl_2$, at 2.5 and 0.02 wt. % vs wet weight of resin, respectively, according to Example 8.

[0424] AP was immobilized on ChiralVision IB-COV-7 resin (3 g) at 2.0 wt. % protein vs wet weight of resin, according to Example 11.

First Extension: 5'-AAAAfGmUfG + mUTP-3'P => 5'-AAAAfGmUfGmU-3'P

[0425] To a 15 mL Falcon tube was added 6.07 mL milli-Q water, 2.25 mL of a 1 M TEAHCl buffer solution (50 mM, pH 7.8), 180 µL of a 100 mM $CoCl_2$ solution (1 mM), 144 µL mUTP-3'P (1.6 mM), and 360 µL 5'-AAAAfGmUfG (800 µM). This solution was maintained at 4 °C for the duration of the extension reaction.

[0426] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 19 h until the reaction had reached completion via the HPLC method described in Example 4, Method A.

[0427] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Second Extension: 5'-AAAAfGmUfGmU + fCTP-3'P => 5'-AAAAfGmUfGmUfC-3'P

[0428] To a reaction solution in a 15 mL Falcon tube containing 600 µM 5'-AAAAfGmUfGmU and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 1200 µM of fCTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0429] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 1.5 h until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0430] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A.

The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Third Extension: 5'-AAAAfGmUfGmUfC + mUTP-3'P => 5'-AAAAfGmUfGmUfCmU-3'P

[0431] To a reaction solution in a 15 mL Falcon tube containing 325 μM 5'-AAAAfGmUfGmUfC and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 650 μM of mUTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0432] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 18.5 h until the reaction had reached completion as determined by the HPLC method described in Example 4 Method A.

[0433] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4 Method A. The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Fourth Extension: 5'-AAAAfGmUfGmUfCmU + fCTP-3'P => 5'-AAAAfGmUfGmUfCmUfC-3'P

[0434] To a reaction solution in a 15 mL Falcon tube containing 250 μM 5'-AAAAfGmUfGmUfCmU and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 500 μM of fCTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0435] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 2.5 h until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0436] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A.

The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Fifth Extension: 5'-AAAAfGmUfGmUfCmUfC + mATP-3'P => 5'-AAAAfGmUfGmUfCmUfCmA-3'P

[0437] To a reaction solution in a 15 mL Falcon tube containing 200 μM 5'-AAAAfGmUfGmUfCmUfC and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 400 μM of mATP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0438] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 7 h until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A

[0439] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Sixth Extension: 5'-AAAAfGmUfGmUfCmUfCmA + mUTP-3'P => 5'-AAAAfGmUfGmUfCmUfCmAmU-3'P

[0440] To a reaction solution in a 15 mL Falcon tube containing 293 μM 5'-AAAAfGmUfGmUfCmUfCmA and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 586 μM of mUTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0441] A BioRad Econo Alpha column was packed with TnT and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 15. The column bed was maintained at 40 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 0.11 mL/min for 5 h until the reaction had reached completion as determined by the LCMS method described in Example 5.

[0442] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the

wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Table 19.1: Extensions of Oligonucleotides Catalyzed by Co-immobilized TnT and iPP in a Flow Reactor

Starting Oligonucleotide	Product Oligonucleotide	Conversion (%)
5'-AAAAfGmUfG	5'-AAAAfGmUfGmU-3'P	+
5'-AAAAfGmUfGmU	5'-AAAAfGmUfGmUfC-3'P	+++
5'-AAAAfGmUfGmUfC	5'-AAAAfGmUfGmUfCmU-3'P	++
5'-AAAAfGmUfGmUfCmU	5'-AAAAfGmUfGmUfCmUfC-3'P	++
5'-AAAAfGmUfGmUfCmUfC	5'-AAAAfGmUfGmUfCmUfCmA-3'P	++
5'-AAAAfGmUfGmUfCmUfCmA	5'-AAAAfGmUfGmUfCmUfCmAmU-3'P	+++
Conversion of nucleotide was determined as percent of total area under curve in the HPLC or LCMS chromatogram and is defined as follows: "+" 0 to 70%, "++" 70 to 90%, "+++" >90%.		

3'P-Dephosphorylation of First Extension: 5'-AAAAfGmUfGmU-3'P => 5'-AAAAfGmUfGmU

[0443] A 15 mL Falcon tube containing a reaction solution comprised of 800 μM 5'-AAAAfGmUfGmU-3'P, residual mUTP-3'P remaining from the RNA oligomer extension step, residual $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0444] A BioRad Econo Alpha column was packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 16. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mUTP-3'P was measured by the HPLC method as described in Example 3.

[0445] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer.

[0446] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the

method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Second Extension: 5'-AAAAfGmUfGmUfC-3'P => 5'-AAAAfGmUfGmUfC

[0447] A 15 mL Falcon tube containing a reaction solution comprised of 600 μM 5'-AAAAfGmUfGmUfC-3'P, residual fCTP-3'P remaining from the RNA oligomer extension step, residual $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0448] A BioRad Econo Alpha column was packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 16. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual fCTP-3'P was measured by the HPLC method as described in Example 3.

[0449] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer.

[0450] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Third Extension: 5'-AAAAfGmUfGmUfCmU-3'P => 5'-AAAAfGmUfGmUfCmU

[0451] A 15 mL Falcon tube containing a reaction solution comprised of 325 μM 5'-AAAAfGmUfGmUfCmU-3'P, residual mUTP-3'P remaining from the RNA oligomer extension step, residual $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0452] A BioRad Econo Alpha column was packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 16. The column bed was maintained at 50 °C via

restive heating jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mUTP-3'P was measured by the HPLC method as described in Example 3.

[0453] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer.

[0454] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Fourth Extension: 5'-AAAAfGmUfGmUfCmUfC-3'P => 5'-AAAAfGmUfGmUfCmUfC

[0455] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

[0456] A 15 mL Falcon tube containing a reaction solution comprised of 325 μM 5'-AAAAfGmUfGmUfCmU-3'P, residual fCTP-3'P remaining from the RNA oligomer extension step, residual $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0457] A BioRad Econo Alpha column was packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 16. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A.

Dephosphorylation of residual fCTP-3'P was measured by the HPLC method as described in Example 3.

[0458] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer.

[0459] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Fifth Extension: 5'-AAAAfGmUfGmUfCmUfCmA-3'P => 5'-AAAAfGmUfGmUfCmUfCmA

[0460] A 15 mL Falcon tube containing a reaction solution comprised of 200 μM 5'-AAAAfGmUfGmUfCmU-3'P, residual mATP-3'P remaining from the RNA oligomer extension step, residual $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0461] A BioRad Econo Alpha column was packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 16. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mATP-3'P was measured by the HPLC method as described in Example 3.

[0462] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer.

[0463] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example

6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7 and adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

Table 19.2: 3'P-Dephosphorylations of Oligonucleotides Catalyzed by Immobilized TnT AP in a Flow Reactor

Starting Oligonucleotide	Product Oligonucleotide	Conversion (%)
5'-AAAAfGmUfGmU-3'P	5'-AAAAfGmUfGmU	+
5'-AAAAfGmUfGmUfC-3'P	5'-AAAAfGmUfGmUfC	+
5'-AAAAfGmUfGmUfCmU-3'P	5'-AAAAfGmUfGmUfCmU	+
5'-AAAAfGmUfGmUfCmUfC-3'P	5'-AAAAfGmUfGmUfCmUfC	+
5'-AAAAfGmUfGmUfCmUfCmA-3'P	5'-AAAAfGmUfGmUfCmUfCmA	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" >95%.		

Example 20: General Procedure for the Co-immobilization of Purified TnT and iPP at 100 g Scale

[0464] TnT enzyme variant (SEQ ID NO: 4) was produced in shake flask and purified as described in Example 1.

[0465] Inorganic pyrophosphatase (iPP) variant (SEQ ID NO: 8) was produced and purified, as described in Example 1.

[0466] Between 100-150 g of ChiralVision IB-HIS-2, pre-loaded with $CoCl_2$, was weighed out and transferred to a 550 mL jacketed reaction vessel fitted with an overhead stirrer. The volume of protein stock containing the desired wt. % of protein vs resin mass was added to a separate flask. Using TEAHCl (500 mM, pH 7.8) as diluent, the volume was diluted to 500 mL and the diluted protein solution transferred to the jacketed reaction vessel containing the resin.

[0467] The jacket temperature was set to 4 °C and the resin was gently stirred at 8 rpm for 20 h until the change in A280 of the immobilization solution became constant. The immobilization solution and resin were drained from the jacketed reaction vessel and the resin was collected on a coarse glass filter via vacuum filtration. The resin was washed with four 250 mL volumes of TEAHCl (50 mM, pH 7.8). The resin was allowed to dry on the filter while maintaining vacuum for approximately ten minutes, then transferred to a sealed plastic storage container and stored at 4 °C.

Example 21: General Procedure for the Immobilization of Purified AP at 100 g Scale

[0468] Alkaline phosphatase (AP) variant (SEQ ID NO: 10) was produced and purified, as described in Example 1.

[0469] Between 100-150 g of ChiralVision IB-COV-7 was weighed out and transferred to a 550 mL jacketed reaction vessel fitted with an overhead stirrer. The volume of protein stock containing the

desired wt. % of protein vs resin mass was added to a separate flask. Using MOPS (20 mM, pH 7.0) as diluent, the volume was diluted to 500 mL and the diluted protein solution transferred to the jacketed reaction vessel containing the resin.

[0470] The jacket temperature was set to 4 °C and the resin was gently stirred at 8 rpm for 20 h until the change in A280 of the immobilization solution became constant. The immobilization solution and resin were drained from the jacketed reaction vessel and the resin collected on a coarse glass filter via vacuum filtration.

[0471] The resin was washed with five 200 mL volumes of TEAHCl (50 mM, pH 7.8) containing 500 mM NaCl, followed by five additional 200 mL wash volumes of TEAHCl (50 mM, pH 7.8). The resin was allowed to dry on the filter while maintaining vacuum for approximately ten minutes, then transferred to a sealed plastic storage container and stored at 4 °C.

Example 22: General Procedure for Enzymatic RNA Oligomer Extensions Using Co-immobilized TnT and iPP in a Multi-Pass Flow Reactor at 1 g Scale

[0472] TnT and IPP were co-immobilized on ChiralVision IB-HIS-2 resin, pre-loaded with $CoCl_2$, at 2.5 and 0.02 wt. % vs wet weight of resin, respectively, according to Example 20. Additional preparations of TnT and iPP co-immobilized on ChiralVision IB-HIS-2 resin at 2.5 and 0.2 wt. % were prepared according to Example 20 as needed.

[0473] A Cytiva XK 16/40 jacketed column (manufacturer part # 28988938) with internal dimensions of 16 mm x 400 mm (80 mL internal volume) fitted with two Cytiva adjustable flow adapters (manufacturer part # 28989876) was packed with 20.3 g of TnT and iPP co-immobilized on ChiralVision IB-HIS-2 resin at 2.5 and 0.02 wt. %, respectively; a portion of TEAHCl (50 mM, pH 7.8) was used to aid in the transfer of resin to the column. The flow adapters were used to compress the resin to give a bed volume of approximately 29 mL. The column bed temperature was maintained with a recirculating water heater at 40-60 °C throughout the course of the extension reaction.

[0474] A 250-1000 mL flask was charged with 50 mM TEAHCl buffer at pH 7.8, 100-3000 μM RNA oligomer, 0.5-2.0 mM $CoCl_2$, and 100-6000 μM NTP-3'P; the reaction reservoir was stored at 4-25 °C for the duration of the flow reaction. This solution was recirculated via peristaltic pump at a flow rate of 0.04-2.0 mL/min first through an inline degasser, then through the temperature-controlled column containing TnT and iPP co-immobilized on IB-HIS-2 at 2.0 and 0.02 wt%, respectively. The endpoint of the reaction was determined by analyzing an aliquot removed from the reservoir via HPLC, as described in Example 4, Method A.

[0475] Upon completion of the reaction, the reaction solution was flushed from the system via peristaltic pump and the column washed with TEAHCl (50 mM, pH 7.8) to collect retained RNA

oligomer. Aliquots of the wash buffer were analyzed by HPLC for the presence of RNA oligomer as described in Example 4, Method A.

Example 23: General Procedure for Enzymatic 3-Dephosphorylation of an RNA Oligomer Using Immobilized Alkaline Phosphatase in a Single-Pass Flow Reactor at 1 g Scale

[0476] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 21. Additional preparations of alkaline phosphatase immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % were prepared according to Example 21 as needed.

[0477] A Cytiva XK 16/40 jacketed column (manufacturer part # 28988938) with internal dimensions of 16 mm x 400 mm (80 mL internal volume) fitted with two Cytiva adjustable flow adapters (manufacturer part # 28989876) was packed with 20.3 g of alkaline phosphatase immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. %; a portion of TEAHCl (50 mM, pH 7.8) was used to aid in the transfer of resin to the column. The flow adapter was used to compress the resin to give a bed volume of approximately 27 mL. The column bed temperature was maintained with a recirculating water heater at 40-60 °C throughout the course of the 3'P-dephosphorylation reaction.

[0478] A 250-1000 mL flask containing the crude extension reaction mix, comprised of 50 mM TEAHCl buffer at pH 7.8, 100-3000 μM RNA oligomer, 0.5-2.0 mM $CoCl_2$, and 200-6000 μM NTP-3'P, was pumped via peristaltic pump at a flow rate of 0.04-5.00 mL/min first through an inline degasser, then through the temperature-controlled column containing alkaline phosphatase immobilized at 2.0 wt. % on ChiralVision IB-COV-7. After passing through the column containing immobilized alkaline phosphatase, the solution was collected in a separate collection vessel. The endpoint of the reaction was determined by analyzing an aliquot removed from the reservoir via HPLC, as described in Example 4, Method A. Residual NTP-3'P dephosphorylation was analyzed by the HPLC method as described in Example 3.

Example 24: Iterative Extension of RNA Oligomer in a Multi-Pass Flow Reactor at 1 g Scale

[0479] TnT enzyme variants SEQ ID NO: 4 and SEQ ID NO: 2 were produced in shake flask and purified as described in Example 1.

[0480] Inorganic pyrophosphatase (iPP) variant (SEQ ID NO: 8) was produced and purified, as described in Example 1.

[0481] Alkaline phosphatase (AP) variant (SEQ ID NO: 10) was produced and purified, as described in Example 1.

[0482] TnT variant (SEQ ID NO: 4) and iPP variant (SEQ ID NO: 8) were co-immobilized on 110 g of ChiralVision IB-HIS-2 resin pre-loaded with $CoCl_2$, at 2.5 and 0.02 wt. % vs wet weight of resin, respectively, according to Example 20.

[0483] TnT variant (SEQ ID NO: 2) and iPP variant (SEQ ID NO: 8) were co-immobilized on 20 g of ChiralVision IB-HIS-2 resin, pre-loaded with $CoCl_2$, at 2.5 and 0.02 wt. % vs wet weight of resin, respectively, according to Example 8.

[0484] AP was immobilized on 110 g ChiralVision IB-COV-7 resin at 2.0 wt. % protein vs wet weight of resin, according to Example 21.

First Extension: 5'-AAAAfGmUfG + mUTP-3'P => 5'-AAAAfGmUfGmU-3'P

[0485] In a 250 mL conical centrifuge tube, a 120 mL reaction stock was prepared containing: 4A0m (3.0 mM), $CoCl_2$ (0.75 mM), SEQ ID NO: 8 (0.5 μM), mUTP-3'P (4.5 mM), SEQ ID NO: 4 (20 μM), TEoAHCl (100 mM), pH 7.8. The tube was capped and incubated 18 h at 40 °C with 100 rpm agitation. A 2 μL aliquot was removed and diluted with 1 mM EDTA for analysis by HPLC according to Example 4, Method A.

Table 24.1. Extension of Oligonucleotide Catalyzed by TnT Variant (SEQ ID NO: 4) and iPP Variant (SEQ ID NO: 8) in Solution at 1 g Scale

Starting Oligonucleotide	Product Oligonucleotide	Conversion (%)
5'-AAAAfGmUfG	5'-AAAAfGmUfGmU-3'P	+
Conversion of nucleotide was determined as percent of total area under curve in the HPLC or LCMS chromatogram and is defined as follows: "+" >90%.		

[0486] The solution was chilled in a 4 °C fridge for 30 minutes and then filtered through a sterile Nalgene 250 mL vacuum filter with a 0.22 μm polyethersulfone membrane that had been pre-washed with RO water.

[0487] A Cytiva XK16-40 column was packed with 50 mL of Ni Sepharose 6 Fast Flow resin in series with two Bio-Rad EconoFit 5 mL columns packed with un-metalated Sepharose 6 Fast Flow resin to prevent Ni leaching. Protein was removed from the reaction mixture by passing through the columns at 5 mL/min. The column was washed with 40 mL of 50 mM TEoAHCl, pH 7.8, giving a final solution volume of 160 mL.

Second Extension: 5'-AAAAfGmUfGmU + fCTP-3'P => 5'-AAAAfGmUfGmUfC-3'P

[0488] To a reaction solution in a 250 mL conical flask containing a 180 mL solution comprised of 2.25 mM 5'-AAAAfGmUfGmU and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 4.5 mM of fCTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0489] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed

resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0490] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 250 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Third Extension: 5'-AAAAfGmUfGmUfC + mUTP-3'P => 5'-AAAAfGmUfGmUfCmU-3'P

[0491] To a reaction solution in a 500 mL flask containing a 271 mL solution comprised of 1.33 mM 5'-AAAAfGmUfGmUfC and 2 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 2.66 mM of mUTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0492] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 2) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour for 24 h.

[0493] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8); these wash volumes were added to the reaction reservoir containing oligomer. The column was removed from the flow reactor and a new Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0494] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 400 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Fourth Extension: 5'-AAAAfGmUfGmUfCmU + fCTP-3'P => 5'-AAAAfGmUfGmUfCmUfC-3'P

[0495] To a reaction solution in a 1000 mL flask containing a 445 mL solution comprised of 809 μM 5'-AAAAfGmUfGmUfCmU and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 1.62 mM of fCTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0496] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0497] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 501 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Fifth Extension: 5'-AAAAfGmUfGmUfCmUfC + mATP-3'P => 5'-AAAAfGmUfGmUfCmUfCmA-3'P

[0498] To a reaction solution in a 1000 mL flask containing a 537 mL solution comprised of 670 μM 5'-AAAAfGmUfGmUfCmUfC and 2 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 1.34 mM of mATP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0499] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0500] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A.

The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 597 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Sixth Extension: 5'-AAAAfGmUfGmUfCmUfCmA + mUTP-3'P => 5'-AAAAfGmUfGmUfCmUfCmAmU-3'P

[0501] To a reaction solution in a 1000 mL flask containing a 637 mL solution comprised of 565 μM 5'-AAAAfGmUfGmUfCmUfCmA and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 1.13 mM of mUTP-3'P. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0502] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0503] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 680 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Seventh Extension: 5'-AAAAfGmUfGmUfCmUfCmAmU + mCQP => 5'-AAAAfGmUfGmUfCmUfCmAmUmC-3'P

[0504] To a reaction solution in a 250 mL conical flask containing a 110 mL solution comprised of 2.0 mM 5'-AAAAfGmUfGmUfCmUfCmAmU and 1 mM $CoCl_2$ in TEAHCl (50 mM, pH 7.8) was added 4.0 mM of mCQP. The reaction solution was maintained at 4 °C throughout the duration of the extension reaction.

[0505] A Cytiva XK 16/40 column was packed with 20.3 g of TnT variant (SEQ ID NO: 4) and iPP co-immobilized on ChiralVision IB-HIS-2 at 2.5 and 0.02 wt. %, respectively, according to the procedure in Example 22. The column bed was maintained at 40 °C via heated water jacket throughout the duration of the experiment. The reaction solution was recirculated through the packed resin bed using a peristaltic pump with a flow rate of 10.2 column volumes per hour until the reaction had reached completion as determined by the HPLC method described in Example 4, Method A.

[0506] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the reaction reservoir giving a final volume of 158 mL, and enzymatic 3'-dephosphorylation with immobilized alkaline phosphatase as outlined below was carried out.

Table 24.2. Extensions of Oligonucleotides Catalyzed by Co-immobilized TnT and iPP in a Multi-Pass Flow Reactor at 1 g Scale

Starting Oligonucleotide	Product Oligonucleotide	Conversion (%)
5'-AAAAfGmUfGmU	5'-AAAAfGmUfGmUfC-3'P	++
5'-AAAAfGmUfGmUfC	5'-AAAAfGmUfGmUfCmU-3'P	+
5'-AAAAfGmUfGmUfCmU	5'-AAAAfGmUfGmUfCmUfC-3'P	++
5'-AAAAfGmUfGmUfCmUfC	5'-AAAAfGmUfGmUfCmUfCmA-3'P	+
5'-AAAAfGmUfGmUfCmUfCmA	5'-AAAAfGmUfGmUfCmUfCmAmU-3'P	++
5'-AAAAfGmUfGmUfCmUfCmAmU	5'-AAAAfGmUfGmUfCmUfCmAmUmC-3'P	++

Conversion of nucleotide was determined as percent of total area under curve in the HPLC or LCMS chromatogram and is defined as follows: "+" 80 to 90%, "++" >90%.

3'P-Dephosphorylation of First Extension: 5'-AAAAfGmUfGmU-3'P => 5'-AAAAfGmUfGmU

[0507] A 250 mL conical flask containing 160 mL of a reaction solution comprised of 2.25 mM 5'-AAAAfGmUfGmU-3'P, residual mUTP-3'P remaining from the RNA oligomer extension step, 0.5 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0508] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mUTP-3'P was measured by the HPLC method as described in Example 3.

[0509] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A.

The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 180 mL.

[0510] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.42 mM $CoCl_2$ according to the method described in Example 7 and was adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Second Extension: 5'-AAAAfGmUfGmUfC-3'P => 5'-AAAAfGmUfGmUfC

[0511] A 500 mL flask containing 250 mL of a reaction solution comprised of 1.44 mM 5'-AAAAfGmUfGmUfC-3'P, residual fCTP-3'P remaining from the RNA oligomer extension step, 0.5 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0512] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual fCTP-3'P was measured by the HPLC method as described in Example 3.

[0513] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 271 mL.

[0514] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.48 mM $CoCl_2$ according to the method described in Example 7 and was adjusted to 2.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Third Extension: 5'-AAAAfGmUfGmUfCmU-3'P => 5'-AAAAfGmUfGmUfCmU

[0515] A 500 mL flask containing 400 mL of a reaction solution comprised of 900 μM 5'-AAAAfGmUfGmUfCmU-3'P, residual mUTP-3'P remaining from the RNA oligomer extension step, 0.5 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0516] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mUTP-3'P was measured by the HPLC method as described in Example 4, Method A.

[0517] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 445 mL.

[0518] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.54 mM $CoCl_2$ according to the method described in Example 7 and was adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Fourth Extension: 5'-AAAAfGmUfGmUfCmUfC-3'P => 5'-AAAAfGmUfGmUfCmUfC

[0519] A 1000 mL flask containing 501 mL of a reaction solution comprised of 719 μM 5'-AAAAfGmUfGmUfCmUfC-3'P, residual fCTP-3'P remaining from the RNA oligomer extension step, 1.0 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0520] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes

per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A.. Dephosphorylation of residual fCTP-3'P was measured by the HPLC method as described in Example 3.

[0521] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 537 mL.

[0522] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.9 mM $CoCl_2$ according to the method described in Example 7 and was adjusted to 2.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Fifth Extension: 5'-AAAAfGmUfGmUfCmUfCmA-3'P => 5'-AAAAfGmUfGmUfCmUfCmA

[0523] A 1000 mL flask containing 597 mL of a reaction solution comprised of 603 µM 5'-AAAAfGmUfGmUfCmUfCmA-3'P, residual mATP-3'P remaining from the RNA oligomer extension step, 2.0 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0524] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mATP-3'P was measured by the HPLC method as described in Example 3.

[0525] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 637 mL.

[0526] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.13 mM $CoCl_2$ according to the method described in Example 7 and was adjusted to 1.0 mM final $CoCl_2$ concentration prior to moving on to the next cycle.

3'P-Dephosphorylation of Sixth Extension: 5'-AAAAfGmUfGmUfCmUfCmAmU-3'P => 5'-AAAAfGmUfGmUfCmUfCmAmU

[0527] A 1000 mL flask containing 680 mL of a reaction solution comprised of 529 μM 5'-AAAAfGmUfGmUfCmUfCmAmU-3'P, residual mUTP-3'P remaining from the RNA oligomer extension step, 1.0 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0528] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mUTP-3'P was measured by the HPLC method as described in Example 3.

[0529] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 713 mL.

[0530] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured according to the method described in Example 7.

3'P-Dephosphorylation of Seventh Extension: 5'-AAAAfGmUfGmUfCmUfCmAmUmC-3'P => 5'-AAAAfGmUfGmUfCmUfCmAmUmC

[0531] A 250 mL flask containing 158 mL of a reaction solution comprised of 1.40 mM 5'-AAAAfGmUfGmUfCmUfCmAmUmC-3'P, residual mCQP remaining from the RNA oligomer extension step, 1.0 mM $CoCl_2$, and 50 mM TEAHCl (pH 7.8) was maintained at room temperature throughout the duration of the reaction.

[0532] A Cytiva XK 16/40 column was packed with 20.3 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. % according to the procedure in Example 23. The column bed was maintained at 50 °C via heated water jacket throughout the duration of the experiment. The reaction solution was pumped through the packed resin bed using a peristaltic pump at a flow rate of 6.8 column volumes per hour until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 4, Method A. Dephosphorylation of residual mCQP was measured by the HPLC method as described in Example 3.

[0533] The system was purged of remaining reaction solution and the column washed with approximately two column volumes of TEAHCl (50 mM, pH 7.8) to remove any retained RNA oligomer from the system. The system wash step was determined to be complete when aliquots of the wash solution contained no RNA oligomer via the HPLC method described in Example 4, Method A. The wash solution containing RNA oligomer was added to the collection vessel containing 3'P-dephosphorylated RNA oligomer for a final collected volume of 178 mL.

[0534] After the wash solution was combined with the collected reaction solution containing RNA oligomer, the residual AP activity of the final reaction solution was measured as described in Example 6. The residual $CoCl_2$ concentration of the final reaction solution was measured at 0.2 mM according to the method described in Example 7.

Table 24.3: 3'P-Dephosphorylations of Oligonucleotides Catalyzed by Immobilized TnT-AP in a Flow Reactor at 1 g Scale

Starting Oligonucleotide	Product Oligonucleotide	Conversion (%)
5'-AAAAfGmUfGmU-3'P	5'-AAAAfGmUfGmU	+
5'-AAAAfGmUfGmUfC-3'P	5'-AAAAfGmUfGmUfC	+
5'-AAAAfGmUfGmUfCmU-3'P	5'-AAAAfGmUfGmUfCmU	+
5'-AAAAfGmUfGmUfCmUfC-3'P	5'-AAAAfGmUfGmUfCmUfC	+
5'-AAAAfGmUfGmUfCmUfCmA-3'P	5'-AAAAfGmUfGmUfCmUfCmA	+
5'-AAAAfGmUfGmUfCmUfCmAmU-3'P	5'-AAAAfGmUfGmUfCmUfCmAmU	+
5'-AAAAfGmUfGmUfCmUfCmAmUmC-3'P	5'-AAAAfGmUfGmUfCmUfCmAmUmC	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" >95%.		

Example 25: RNA Oligomer Lyophilization and Desalting Procedure

[0535] The solution containing a crude mixture of RNA oligomer, residual $CoCl_2$, residual NTP-3'P, residual nucleoside, and buffer salts was transferred to a LyoGuard tray and placed in the lyophilizer. The cold shelf temperature in the lyophilizer was set a temperature of -40 °C and the crude RNA oligomer solution was allowed to completely freeze over a period of 18 h.

[0536] Once completely frozen, the cold shelf temperature was gradually increased from -40 °C to 10 °C over a 5 h period. After reaching a cold shelf temperature of 10 °C, the crude RNA oligomer mixture was allowed to dry for an additional 48 h. Upon completion of drying, the crude RNA oligomer mixture was resuspended in 30 mL milli-Q water and passed through a 0.4 μm pre-washed vacuum filter frit.

[0537] Two HiPrep 26/10 pre-packed desalting columns (manufacturer part # 17508701) were connected in series on an AKTA Start with UV and conductance monitoring and conditioned with 10 column volumes of milli-Q water pumped at a flow rate of 9.0 mL/min. One 15 mL fraction of crude RNA oligomer was loaded onto the column at a flow rate of 2.0 mL/min and then fractionated using milli-Q water pumped at a constant flow rate of 2.0 mL/min into 5 mL fractions.

[0538] Fractions which contained RNA oligomer but had no salt as measured by the conductivity meter were pooled in a collection flask; fractions containing RNA oligomer and salt were pooled separately. An aliquot from each fraction was evaluated using the HPLC method described in Example 4, Method A. The HiPrep 26/10 desalting columns were washed rigorously with milli-Q water and the pooled fractions containing RNA oligomer and salt were passed through the columns again at a flow rate of 2.0 mL/min. Fractions containing RNA oligomer but had no salt as measured by the conductivity meter were pooled with initial collection of no salt fractions.

[0539] The collection of pooled fractions containing RNA oligomer without salt were frozen and lyophilized using the above-described method.

Example 26: Higher TnT Loading on IB-HIS-2 and Effect on Activity and Recyclability

[0540] TnT enzyme variant (SEQ ID NO: 6) was produced and purified according to Example 1.

[0541] Two preparations of immobilized TnT on approximately 30 mg of ChiralVision IB-HIS-2 resin were produced according to Example 8 with a target weight percent loading of 2.5 and 5.0 vs wet weight of resin.

Table 26.1: Immobilization of TnT(SEQ ID NO: 6) on IB-HIS-2 at 2.0 and 5.0 wt.% vs Wet Weight of Resin

Targeted TnT Weight Percent Loading	Percent of Target Loading
2.5	+
5.0	+
Levels of immobilization are determined by ratio of A280 vs negative control and defined as follows: "+" 90 to 100%.	

Extension of AAAAfGmUfG with mATP-3'P and Recyclability

[0542] A reaction stock was prepared, consisting of: AAAAfGmUfG (1.0 mM), mATP-3'P (1.5 mM), $CoCl_2$ (1.0 mM), TEoAHCl (50 mM), pH 7.8. The reaction stock was added to wells containing

resin at a volume of 10 µL per mg of resin and incubated for 2 h with 500 rpm agitation at 50 °C. A 2 µL aliquot was removed and diluted with EDTA for analysis by HPLC according to Example 4, Method A.

[0543] To test recyclability, the supernatant was removed from each resin sample and the resin was washed once by incubating in 300 µL 50 mM TEoAHCl (pH 7.8) for 10 minutes at room temperature on benchtop shaker. The buffer was removed and fresh reaction stock containing AAAAfGmUfG was added to each sample at a volume of 10 µL per mg of resin and incubated for 2 h with 500 rpm agitation at 50 °C.

[0544] The reaction supernatant was removed from each resin sample and the resin was washed once by incubating in 300 µL 50 mM TEoAHCl (pH 7.8) for 10 minutes at room temperature on benchtop shaker.

[0545] The reaction was run twice more, each time using fresh reaction stock containing AAAAfGmUfG and collecting a 2 µL aliquot after 2 hours of reaction for analysis by HPLC according to Example 4, Method A. Conversion of AAAAfGmUfG to AAAAfGmUfGmA-3'P is shown in **Table 26.2**.

Table 26.2: Activity and Recyclability of TnT SEQ ID NO: 6 Immobilized on IB-HIS-2 Resin at 2.5 and 5.0 Weight Percent

Target Weight Percent Loading (%)	Cycle	Percent Conversion to AAAAfGmUfGmA-3'P
5	1	+++
2.5	1	++
5	2	++
2.5	2	+
5	3	++
2.5	3	+
Levels of immobilization are determined by ratio of A280 vs negative control and defined as follows: "+" 15 to 30%, "++" 30 to 50%, "+++" 50 to 70%		

Example 27: Removal of Leached Alkaline Phosphatase Using Immobilized Metal Affinity Chromatography (IMAC) Resin to Reduce Byproduct Formation

[0546] TnT enzyme variants SEQ ID NO: 2 and SEQ ID NO: 4 were produced and purified according to Example 1.

[0547] iPP enzyme variant (SEQ ID NO: 8) was produced and purified according to Example 1.

[0548] Alkaline phosphatase enzyme variant (SEQ ID NO: 10) was produced and purified according to Example 1.

[0549] A sample containing the oligonucleotide AAAAfGmUfGmUfC, produced from a prior TnT catalyzed extension, was found to be contaminated with AP variant SEQ ID NO: 10. Immobilized

metal affinity chromatograph (IMAC) resin, that may be loaded with either Ni(II) or Co(II), was used to remove the contaminating SEQ ID NO: 10.

[0550] A His GraviTrap™ gravity flow column prepacked with Ni Sepharose™ 6 Fast Flow resin, purchased from Cytiva, was washed and conditioned with TEoAHCl (pH 7.8, 50 mM) according to the provided instructions. A 500 µL aliquot was removed from the sample containing AAAAfGmUfGmUfC. The remainder was then filtered through the column according to the instructions provided and a 500 µL aliquot was saved from the filtered sample.

[0551] The $CoCl_2$ content in the filtered and unfiltered aliquots was measured according to the residual $CoCl_2$ assay outlined in Example 7 and additional $CoCl_2$ was added from a 100 mM stock solution in RO water, to bring the concentration in both aliquots back to 1 mM. Nucleotide (mUTP-3'P) was also added to both the filtered and unfiltered aliquots to bring the mUTP-3'P concentration up to 2 equivalents vs oligonucleotide concentration.

[0552] Resin (IB-HIS-2) which had been purchased from un-metalated from ChiralVision, charged with $CoCl_2$, and co-loaded with 5 wt. % TnT (SEQ ID NO: 2 or SEQ ID NO: 4) and 0.02 wt. % iPP, was weighed out in four Eppendorf tubes in 5 - 15 mg amounts.

[0553] The filtered sample aliquot was transferred to two of the Eppendorf tubes containing resin (10 µL sample per mg resin), one tube with TnT variant SEQ ID NO: 2 and the other with TnT variant SEQ ID NO: 4. The unfiltered sample aliquot was transferred to the other two Eppendorf tubes containing resin (10 µL sample per mg resin).

[0554] The samples were incubated at 50 °C with 500 rpm agitation, and 5 µL aliquots were removed at 3.5 hrs and 18 hrs for analysis by HPLC as described in Example 4, Method A. The conversion of 5'-AAAAfGmUfGmUfC to 5'-AAAAfGmUfGmUfCmU-3'P is shown in **Table 2.1**. The conversion to overextended byproducts is calculated using the area under curve (AUC) of all peaks in the chromatogram with a higher retention time than the desired product AAAAfGmUfGmUfCmU-3'P.

Table 27.1: Conversion of AAAAfGmUfGmUfC to AAAAfGmUfGmUfCmU-3'P after filtering through IMAC resin to remove contaminating alkaline phosphatase.

Time (hours)	TnT Variant	Percent Conversion to AAAAfGmUfGmUfCmU-3'P	Percent Conversion to Overextended Byproducts
3.5	SEQ ID NO: 2	++	++
3.5	SEQ ID NO: 2	+++	+
3.5	SEQ ID NO: 4	++	++
3.5	SEQ ID NO: 4	+++	+
18	SEQ ID NO: 2	+	+++
18	SEQ ID NO: 2	+++	++
18	SEQ ID NO: 4	+	+++
18	SEQ ID NO: 4	+++	++

Percent conversion to AAAAfGmUfGmUfCmU-3'P is determined by ratio of AUC Product vs AUC substrate and byproducts in the HPLC chromatogram and defined as follows: "+" 0 to 10%, "++" 20 to 30%, "+++" 50 to 80%. Percent conversion to overextended byproducts is determined by ratio of AUC byproducts vs AUC substrate and AAAAfGmUfGmUfCmU-3'P in the HPLC chromatogram and defined as follows: "+" 0 to 10%, "++", 15 to 30%, "+++" 50 to 80%

Example 28: analysis of an enzymatically elongated oligonucleotide

[0555] An enzymatically elongated oligonucleotide as manufactured by the systems and methods described herein, and subjected to LCMS analysis as described in Example 5.

[0556] Species with m/z corresponding to n+1 and n-1 was observed (**FIG. 16**). Species corresponding an oxidation product of a phosphorothioate bond were also observed (**FIG. 16**). Depurination and depyrimidation events, and oligonucleotides comprising cNet, methyl, acetyl, or isobutyryldiaminopurine in place of the intended nucleotide were also not observed (**FIG. 16**).

[0557] 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.

[0558] For all purposes, 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.

CLAIMS

What is claimed is:

1. A method of template-free synthesis of an oligonucleotide, comprising:

elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety;

separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase;

removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed.

2. The method of claim 1, wherein the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

3. The method of claim 1 or 2, wherein the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase.

4. The method of claim 3, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

5. The method of claim 3 or 4, further comprising separating the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase.

6. The method of any one of claims 1-5, further comprising repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety.

7. The method of claim 6, wherein a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety.

8. The method of claim 6 or 7, wherein at least two different types of transferases are used in separate cycles.

9. The method of any one of claims 1-8, wherein the elongating occurs in a first reaction chamber.

10. The method of any one of claims 1-9, wherein the removing the 3′ blocking moiety occurs in a second reaction chamber.

11. A method of template-free synthesis of an oligonucleotide, comprising:

in a first reaction chamber, elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety;

flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber, wherein the transferase is retained in the first reaction chamber;

in the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed; and

flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the second reaction chamber into the first reaction chamber.

12. The method of claim 11, wherein the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

13. The method of claim 11 or 12, wherein the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase.

14. The method of claim 13, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

15. The method of any one of claims 13-14, wherein the hydrolase is retained in the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows out of the second reaction chamber.

16. The method of any one of claims 11-15, further comprising repeating the method for one or more cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety.

17. The method of claim 16, wherein a plurality of predetermined nucleotides are used to elongate the oligonucleotide, thereby generating an elongated oligonucleotide having a predetermined sequence, wherein each cycle after a first cycle attaches a single nucleotide to a previously elongated oligonucleotide without the 3′ blocking moiety.

18. The method of claim 16 or 17, wherein at least two different types of transferases are used in separate cycles.

19. The method of any one of claims 11-18, wherein the first reaction chamber and the second reaction chamber are connected to each other through one or more conduits, and the elongated oligonucleotide comprising the 3′ blocking moiety and the elongated oligonucleotide without the 3′ blocking moiety flows through at least a portion of the one or more conduits.

20. The method of any one of claims 11-19, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more pumps.

21. The method of any one of claims 11-20, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more valves.

22. The method of any one of claims 11-21, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the first reaction chamber.

23. The method of any one of claims 11-22, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the second reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the second reaction chamber.

24. The method of any one of claims 11-23, wherein the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an outlet of the second reaction chamber.

25. The method of any one of claims 11-24, wherein the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the first reaction chamber comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety through an inlet of the first reaction chamber.

26. The method of any one of claims 11-25, wherein flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into the first reaction chamber comprises:

flowing the elongated oligonucleotide without the 3′ blocking moiety from the second reaction chamber into one or more reservoirs, and

flowing the elongated oligonucleotide without the 3′ blocking moiety from the one or more reservoirs into the first reaction chamber.

27. A method of template-free synthesis of an oligonucleotide, comprising, in a plurality of reaction chambers:

in at least a first reaction chamber, elongating an oligonucleotide in solution by attaching a

first nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a first transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety;

flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution from the at least the first reaction chamber into at least a second reaction chamber, wherein the first transferase is retained in the at least the first reaction chamber;

in the at least the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed;

flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the at least the second reaction chamber into at least a third reaction chamber comprising a second transferase; and

in the at least the third reaction chamber, elongating the elongated oligonucleotide without the 3′ blocking moiety the solution by attaching a second nucleotide comprising a 3′ blocking moiety to the elongated oligonucleotide without the 3′ blocking moiety using the second transferase to make a further elongated oligonucleotide comprising the 3′ blocking moiety of the second nucleotide.

28. The method of claim 27, wherein the first transferase and the second transferase are different types of transferases.

29. The method of claim 27 or 28, wherein the first nucleotide and the second nucleotide are preselected types of nucleotides, and/or wherein the first nucleotide and the second nucleotide are different types of nucleotides.

30. The method of any one of claims 27-29, wherein the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety using a hydrolase.

31. The method of claim 30, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

32. The method of claim 30 or 31, wherein the hydrolase is retained in the at least the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows from the second reaction chamber into the at least the third reaction chamber.

33. The method of any one of claims 27-32, wherein the first reaction chamber, the second reaction chamber, and the third reaction chamber are connected through a plurality of conduits, wherein:

the elongated oligonucleotide comprising the 3′ blocking moiety flows from the at least the first reaction chamber into at least a second reaction chamber through a first portion of the plurality of conduits; and

the elongated oligonucleotide without the 3′ blocking moiety flows from the at least the

second reaction chamber into at least a third reaction chamber through a second portion of the plurality of conduits.

34. The method of any one of claims 27-33, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more pumps.

35. The method of any one of claims 27-34, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety into the at least the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety into the at least the third reaction chamber is controlled by one or more valves.

36. The method of any one of claims 1-35, wherein the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog.

37. The method of claim 36, wherein the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety.

38. The method of any one of claims 3-10, 13-26, and 30-37, wherein the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution.

39. The method of any one of claims 3-10, 13-26, and 30-38, wherein the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

40. The method of claim 39, wherein the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

41. The method of any one of claims 3-10, 13-26, and 30-40, wherein the hydrolase is immobilized on a solid support.

42. The method of any one of claims 1-41, wherein the transferase comprises a polymerase from the DNA polymerase X family; a template independent transferase, preferably a terminal deoxynucleotidyl transferase (TdT); or an ssRNA ligase.

43. The method of any one of claims 1-42, wherein the transferase is immobilized on a solid support.

44. The method of any one of claims 1-43, wherein elongating produces an inorganic pyrophosphate byproduct.

45. The method of claim 44, further comprising degrading the inorganic pyrophosphate using a pyrophosphatase, preferably using an inorganic pyrophosphatase.

46. The method of claim 45, wherein the pyrophosphatase is immobilized on a solid support.

47. The method of claim 45 or 46, wherein the elongating and the degrading occur within the same reaction chamber.

48. The method of claim 47, wherein the transferase and the pyrophosphatase are fused together, or wherein the transferase and the pyrophosphatase are immobilized on the same solid support, or wherein the transferase and the pyrophosphatase are immobilized on different solid supports.

49. The method of claim 45 or 47, wherein the transferase is immobilized on a solid support and the pyrophosphatase is in the solution.

50. The method of claim 49, wherein the pyrophosphatase is retained in the reaction chamber comprising the transferase, wherein the reaction chamber comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide.

51. The method of any one of claims 45-50, wherein the elongating and the degrading occur within different reaction chambers.

52. The method of claim 51, comprising flowing the elongated oligonucleotide comprising the 3′ blocking moiety and the inorganic pyrophosphate byproduct, in the solution, from a reaction chamber comprising the transferase into a reaction chamber comprising the pyrophosphatase, followed by flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reaction chamber comprising the pyrophosphatase into a reaction chamber in which the 3′ blocking moiety is removed from the elongated oligonucleotide comprising the 3′ blocking moiety.

53. The method of any one of claims 10-52, wherein the first reaction chamber or the second reaction chamber is a column.

54. The method of claim 53, wherein the first reaction chamber comprises
a fixed bed comprising the transferase immobilized on a solid support; or
a fluidized bed comprising the transferase immobilized on a solid support; or
a filter that prevents passage of the transferase and allows passage of the oligonucleotide.

55. The method of claim 53 or 54, wherein the second reaction chamber comprises
a fixed bed comprising the hydrolase immobilized on a solid support; or
a fluidized bed comprising the hydrolase immobilized on a solid support; or
a filter that prevents passage of hydrolase and allows passage of the oligonucleotide.

56. The method of any one of claims 10-52, wherein the first reaction chamber or the second reaction chamber is a batch reaction chamber.

57. The method of claim 56, wherein the batch reaction chamber comprises an impeller.

58. The method of claim 55, wherein the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support.

59. The method of any one of claims 56-58, wherein the second reaction chamber comprises a rotating bed reactor comprising hydrolase immobilized on a solid support.

60. The method of any one of claims 1-58, further comprising separating unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide.

61. The method of claim 60, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography, preferably size exclusion chromatography, reverse phase chromatography, or ion exchange chromatography.

62. The method of claim 60, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis or tangential flow filtration.

63. The method of any one of claims 60-62, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides, or only after elongating the oligonucleotide by four or more nucleotides.

64. The method of any one of claims 1-63, wherein the 3′ blocking moiety is a phosphate moiety.

65. The method of any one of claims 1-64, wherein the nucleotide comprising the 3′ blocking moiety comprises a ribonucleotide or a deoxyribonucleotide, or further comprises a 2′ modification, preferably 2′-F or 2′-O-methyl.

66. The method of any one of claims 1-65, wherein the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate.

67. A method of template-free synthesis of an oligonucleotide, comprising:

flowing an oligonucleotide, in a solution, from a reagent reservoir into a first reaction chamber comprising a transferase;

in the first reaction chamber, elongating the oligonucleotide by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using the transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety;

flowing the elongated oligonucleotide comprising the nucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber comprising a hydrolase, wherein the transferase is retained in the first reaction chamber;

in the second reaction chamber, removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to make an elongated

oligonucleotide without the 3′ blocking moiety; and

flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, into the reagent reservoir.

68. The method of claim 67, comprising mixing the oligonucleotide and the nucleotides comprising a 3′-blocking moiety in the reservoir.

69. The method of claim 67 or 68, further comprising flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety, in the solution, through a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety before flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety into the reagent reservoir.

70. The method of any one of claims 67-69, further comprising flowing the oligonucleotide without the 3′ blocking moiety into a product reservoir before flowing the oligonucleotide without the 3′ blocking moiety into the reagent reservoir.

71. The method of any one of claims 67-70, further comprising replacing or cleaning the reagent reservoir before flowing the elongated oligonucleotide without the 3′ blocking moiety into the reagent reservoir.

72. The method of any one of claims 67-71, comprising repeating the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety.

73. The method of claim 72, further comprising flowing buffer without the oligonucleotide through the system between cycles.

74. A method of template-free synthesis of an oligonucleotide, comprising:

(a) elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to an oligonucleotide transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety, the elongating comprising subjecting the oligonucleotide to one or more elongation flow pathway cycles comprising:

flowing the oligonucleotide and nucleotides comprising a 3′ blocking moiety from a reagent reservoir to a first reaction chamber comprising a transferase, and

flowing the oligonucleotide from the first reaction chamber to the reservoir; and

(b) removing the 3′ blocking moiety from the oligonucleotide in the solution to make an elongated oligonucleotide without the 3′ blocking moiety, comprising subjecting the oligonucleotide to one or more deblocking flow pathway cycles comprising:

flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent

reservoir to a second reaction chamber comprising a hydrolase, and

flowing the elongated oligonucleotide from the second reaction chamber to the reagent reservoir.

75. A method of template-free synthesis of an oligonucleotide, comprising:

(a) elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to an oligonucleotide transferase to make an elongated oligonucleotide comprising the 3′ blocking moiety, the elongating comprising subjecting the oligonucleotide to one or more elongation flow pathway cycles comprising:

flowing the oligonucleotide and 3′-blocked nucleotides from a reagent reservoir to a first reaction chamber comprising a transferase, and

flowing the oligonucleotide from the first reaction chamber to the reservoir;

(b) flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent reservoir to a second reaction chamber comprising a hydrolase; and

(c) removing the 3′ blocking moiety from the oligonucleotide in the solution to make an elongated oligonucleotide without the 3′ blocking moiety, comprising subjecting the oligonucleotide to one or more deblocking flow pathway cycles comprising:

flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the second reaction chamber to a product reservoir, and

flowing the elongated oligonucleotide from the product reservoir to the second reaction chamber.

76. The method of claim 74 or 75, wherein the elongating comprises subjecting the oligonucleotide to a plurality of elongation flow path cycles.

77. The method of any one of claims 74-76, wherein the removing comprises subjecting the oligonucleotide to a plurality of deblocking flow pathway cycles.

78. The method of any one of claims 74-77, comprising separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety.

79. The method of claim 78, wherein the separating comprises subjecting the oligonucleotide to one or more purification flow pathway cycles comprising:

flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety from the reagent reservoir or the product reservoir to a purification chamber configured to separate the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety, and

flowing the elongated oligonucleotide without the 3′ blocking moiety or the elongated

oligonucleotide comprising the 3′ blocking moiety from the purification chamber to the reagent reservoir or the product reservoir.

80. The method of claim 78 or 79, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography, preferably size exclusion chromatography, reverse phase chromatography, or ion exchange chromatography.

81. The method of claim 78 or 79, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis or tangential flow filtration.

82. The method of any one of claims 78-81, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides, or only after elongating the oligonucleotide by four or more nucleotides.

83. The method of any one of claims 74-81, comprising repeating the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety.

84. A method of template-free synthesis of an oligonucleotide, comprising:

in first reaction chamber, elongating an oligonucleotide in a solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase immobilized on a solid support suspended in the solution to make an elongated oligonucleotide comprising the 3′ blocking moiety;

flowing the elongated oligonucleotide comprising the 3′ blocking moiety in the solution to a second reaction chamber, wherein the transferase is retained in the first reaction chamber;

in the second reaction chamber, removing the 3′ blocking moiety from the an elongated oligonucleotide comprising the 3′ blocking moiety in the solution using a hydrolase immobilized on a solid support suspended in the solution to make an elongated oligonucleotide without the 3′ blocking moiety; and

flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, into the first reaction chamber.

85. The method of claim 84, comprising separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide without the 3′ blocking moiety or the elongated oligonucleotide comprising the 3′ blocking moiety.

86. The method of claim 85, wherein:

the separating comprises flowing the elongated oligonucleotide comprising the 3′ blocking moiety from the first reaction chamber to a purification chamber configured to separate unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety; or

the separating comprises flowing the elongated oligonucleotide without the 3′ blocking

moiety from the second reaction chamber to a purification chamber configured to separate unreacted nucleotides or reaction byproducts from the elongated oligonucleotide without the 3′ blocking moiety.

87. The method of any one of claims 84-86, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using liquid chromatography.

88. The method of claim 87, wherein the liquid chromatography comprises size exclusion chromatography, reverse phase chromatography, or ion exchange chromatography.

89. The method of any one of claims 84-86, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide using dialysis, or tangential flow filtration.

90. The method of any one of claims 84-89, wherein the unreacted nucleotides or reaction byproducts are separated from the oligonucleotide after elongating the oligonucleotide by four or more nucleotides, or only after elongating the oligonucleotide by four or more nucleotides.

91. The method of any one of claims 84-89, comprising repeating the method for at least two cycles to further elongate the elongated oligonucleotide without the 3′ blocking moiety.

92. The method of any one of claims 67-91, wherein the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

93. The method of any one of claims 67-92, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

94. The method of any one of claims 67-93, wherein the hydrolase is retained in the second reaction chamber when the elongated oligonucleotide without the 3′ blocking moiety flows out of the second reaction chamber.

95. The method of any one of claims 67-94, wherein the first reaction chamber and the second reaction chamber are connected to each other through one or more conduits, and the elongated oligonucleotide comprising the 3′ blocking moiety and the elongated oligonucleotide without the 3′ blocking moiety flows through at least a portion of the one or more conduits.

96. The method of any one of claims 67-95, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more pumps.

97. The method of any one of claims 67-96, wherein the flowing of the elongated oligonucleotide comprising the 3′ blocking moiety to the second reaction chamber and/or the flowing of the elongated oligonucleotide without the 3′ blocking moiety to the first reaction chamber is controlled by one or more valves.

98. The method of any one of claims 67-97, wherein the nucleotide comprising the 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog.

99. The method of claim 98, wherein the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety.

100. The method of any one of claims 67-99, wherein the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution.

101. The method of any one of claims 67-100, wherein the hydrolase removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

102. The method of claim 101, wherein the hydrolase removes three 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

103. The method of any one of claims 67-102, wherein the hydrolase is immobilized on a solid support.

104. The method of any one of claims 67-103, wherein the transferase is a polymerase from the DNA polymerase X family; a template independent transferase, preferably a terminal deoxynucleotidyl transferase (TdT); or an ssRNA ligase.

105. The method of any one of claims 67-104, wherein the transferase is immobilized on a solid support.

106. The method of any one of claims 67-105, wherein elongating produces an inorganic pyrophosphate byproduct.

107. The method of claim 106, further comprising degrading the inorganic pyrophosphate using a pyrophosphatase, preferably an inorganic pyrophosphatase.

108. The method of claim 107, wherein the pyrophosphatase is immobilized on a solid support.

109. The method of any one of claims 107-108, wherein the elongating and the degrading occur within the same reaction chamber.

110. The method of claim 109, wherein the transferase and the inorganic pyrophosphatase are fused together, or wherein the transferase and the pyrophosphatase are immobilized on the same solid support or different solid supports.

111. The method of claim 107 or 109, wherein the transferase is immobilized on a solid support and the pyrophosphatase is in the solution.

112. The method of claim 111, wherein the pyrophosphatase is retained in the reaction chamber comprising the transferase, wherein the reaction chamber comprises a filter that prevents passage of the pyrophosphatase and allows passage of the oligonucleotide.

113. The method of any one of claims 107-112 wherein the elongating and the degrading occur within different reaction chambers.

114. The method of any one of claims 67-113, wherein the first reaction chamber or the second reaction chamber is a column.

115. The method of claim 114, wherein the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support, or comprises a fluidized bed comprising the transferase immobilized on a solid support, or comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide.

116. The method of claim 114 or 115, wherein the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support or a fluidized bed comprising the hydrolase immobilized on a solid support, or a filter that prevents passage of hydrolase and allows passage of the oligonucleotide.

117. The method of any one of claims 67-106, wherein the first reaction chamber or the second reaction chamber is a batch reaction chamber.

118. The method of claim 117, wherein the batch reaction chamber comprises an impeller or a rotating bed reactor comprising the transferase immobilized on a solid support.

119. The method of claim 117 or 118, wherein the second reaction chamber comprises a rotating bed reactor comprising hydrolase immobilized on a solid support.

120. The method of any one of claims 67-119, wherein the 3′ blocking moiety is a phosphate moiety.

121. The method of any one of claims 67-120 wherein the nucleotide comprising the 3′ blocking moiety is a ribonucleotide or a deoxyribonucleotide, or further comprises a 2′ modification, preferably 2′-F or 2′-O-methyl.

122. The method of any one of claims 67-121, wherein the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate.

123. The method of any one of claims 67-122, wherein the oligonucleotide comprises a 5′ modification.

124. A method of template-free synthesis of an oligonucleotide, comprising:

elongating an oligonucleotide in solution by attaching a nucleotide comprising a 3′ blocking moiety to the oligonucleotide using a transferase in the solution to make an elongated oligonucleotide

comprising the 3′ blocking moiety;

separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase;

removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety to make an elongated oligonucleotide without the 3′ blocking moiety, the elongated oligonucleotide comprising the 3′ blocking moiety being in solution when the 3′ blocking moiety is removed.

125. The method of claim 124, wherein separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase comprises flowing the solution through a column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety.

126. The method of claim 125, wherein the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety is a liquid chromatography column.

127. The method of claim 125 or 126, wherein the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column, or comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide comprising the 3′ blocking moiety.

128. The method of any one of claims 125-127, comprising flowing the oligonucleotide and the transferase, in the solution, from a reagent reservoir to the column that retains the transferase, and flowing the oligonucleotide comprising the 3′ blocking moiety, in the solution, from the column that retains the transferase to the reagent reservoir.

129. The method of any one of claims 124-128, wherein removing the 3′ blocking moiety from the elongated oligonucleotide comprising the 3′ blocking moiety comprises adding a hydrolase to the solution after separating the elongated oligonucleotide comprising the 3′ blocking moiety from the transferase.

130. The method of claim 129, further comprising separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety.

131. The method of claim 130, wherein separating the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety from the hydrolase comprises flowing the solution through a column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety.

132. The method of claim 131, wherein the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column.

133. The method of claim 131 or 132, wherein the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety is a size exclusion column, an affinity column, an ion exchange column, or a reverse phase column, or comprises a filter that prevents passage of the hydrolase and allows passage of the elongated oligonucleotide without the 3′ blocking moiety.

134. The method of any one of claims 129-133, comprising flowing the elongated oligonucleotide without the 3′ blocking moiety and the hydrolase, in the solution, from a reagent reservoir to the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety, and flowing the elongated oligonucleotide without the 3′ blocking moiety, in the solution, from the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety to the reagent reservoir.

135. The method of any one of claims 129-134, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

136. The method of any one of claims 125-135, comprising replacing or cleaning the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety or the column that separates the hydrolase from the elongated oligonucleotide without the 3′ blocking moiety.

137. The method of claim 136, wherein the cleaning comprises flowing a buffer through the column to elute the transferase or the hydrolase.

138. The method of any one of claims 124-137, further comprising separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety.

139. The method of claim 138, wherein separating unreacted nucleotides or reaction byproducts in the solution from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety comprises flowing the solution through a column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety.

140. The method of claim 139, wherein the column that separates the unreacted nucleotides or reaction byproducts from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety is a liquid chromatography column, preferably a size exclusion column, a reverse phase column, or an ion exchange column.

141. The method of claim 138, wherein the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety using dialysis or tangential flow filtration.

142. The method of any one of claims 138-141, wherein the unreacted nucleotides or reaction byproducts are separated from the elongated oligonucleotide comprising the 3′ blocking moiety or the elongated oligonucleotide without the 3′ blocking moiety after elongating the oligonucleotide by four or more nucleotides, or only after elongating the oligonucleotide by four or more nucleotides.

143. The method of any one of claims 124-142, wherein the nucleotide comprising the 3′ blocking moiety is a preselected type of nucleotide.

144. The method of any one of claims 124-143, wherein the nucleotide comprising a 3′ blocking moiety is a nucleotide triphosphate comprising a 3′ blocking moiety or an analog thereof comprising a 5′ phosphate analog.

145. The method of claim 144, wherein the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety.

146. The method of any one of claims 125-145, wherein the hydrolase removes the 3′ blocking moiety from unreacted nucleotides in the solution, and/or removes one or more 5′ phosphate moieties and/or 5′ phosphate analogs from unreacted nucleotides in the solution.

147. The method of any one of claims 124-146, wherein the transferase is a polymerase from the DNA polymerase X family; a template independent transferase, preferably a terminal deoxynucleotidyl transferase (TdT); or an ssRNA ligase.

148. The method of any one of claims 124-147, wherein elongating produces an inorganic pyrophosphate byproduct.

149. The method of claim 124-148, further comprising degrading the inorganic pyrophosphate using a pyrophosphatase, preferably an inorganic pyrophosphatase.

150. The method of claim 149 or 149, wherein the transferase and the inorganic pyrophosphatase are fused together.

151. The method of any one of claims 139-149, wherein the column that separates the transferase from the elongated oligonucleotide comprising the 3′ blocking moiety further separates the pyrophosphatase from the elongated oligonucleotide comprising the 3′ blocking moiety.

152. The method of any one of claims 124-151, wherein the 3′ blocking moiety is a phosphate moiety.

153. The method of any one of claims 124-152, wherein the nucleotide comprising the 3′ blocking moiety is a ribonucleotide or a deoxyribonucleotide.

154. The method of any one of claims 124-153, wherein the nucleotide comprising the 3′ blocking moiety comprises a 2′ modification, preferably 2′-F or 2′-O-methyl.

155. The method of any one of claims 124-154, wherein the nucleotide comprising the 3′ blocking moiety comprises a nucleoside 5′-(α-P-thio)phosphate.

156. The method of any one of claims 124-155, wherein the oligonucleotide comprises a 5′ modification.

157. The method of any one of claims 1-66, wherein the oligonucleotide comprises a 5′ modification.

158. The method of any one of claims 1-157, wherein the oligonucleotide is at least 4 nucleotides in length.

159. The method of any one of claims 1-158, comprising synthesizing the oligonucleotide prior to elongating the oligonucleotide with the transferase.

160. The method of claim 159, wherein synthesizing the oligonucleotide comprises attaching a nucleotide donor to a nucleotide acceptor having a 3′-OH group using a primase.

161. The method of claim 160, wherein the nucleotide acceptor is a nucleotide monomer.

162. The method of claim 160, wherein the nucleotide acceptor is a nucleotide polymer, preferably a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, or 7-mer.

163. The method of any one of claims 160-162, wherein the nucleotide donor is a nucleotide monomer.

164. The method of any one of claims 160-163, wherein the nucleotide donor comprises a 3′-blocking moiety.

165. The method of claim 164, wherein the 3′-blocking moiety of the nucleotide donor is a phosphate.

166. The method of claim 164 or 165, comprising removing the 3′-blocking moiety of the nucleotide donor attached to the nucleotide acceptor to form an elongated nucleotide acceptor.

167. The method of claim 166, wherein the 3′-blocking moiety is removed from the nucleotide donor using a hydrolase.

168. The method of any one of claims 159-167, wherein making the oligonucleotide comprises one or more primase extension cycles, comprising:

attaching a nucleotide donor comprising a 3′-blocking moiety to a nucleotide acceptor having

a 3′-OH group using a primase to make a 3′ -blocked donor-acceptor oligonucleotide;

inactivating the primase or separating the primase from the 3′ -blocked donor-acceptor oligonucleotide;

removing the 3′-blocking moiety from the 3′ -blocked donor-acceptor oligonucleotide;

wherein each cycle uses a new nucleotide donor.

169. The method of any one of claims 42, 104, or 144, wherein when the transferase comprises a single strand RNA ligase, the nucleotide comprising the 3′ blocking moiety comprises a conjugate moiety, a reactive group, or a linker.

170. The method of claim 169, wherein nucleotide comprising the 3′ blocking moiety comprises the conjugate moiety, and the conjugate moiety comprises a carbohydrate, a lipid or a lipophilic group, a sterol, a drug, a hormone, a polymer, a protein, a peptide, a toxin, a vitamin, or a combination thereof.

171. The method of claim 169 or 170, wherein the oligonucleotide comprises a 5′ blocking moiety other than a 5′-phosphate moiety, or a 5′-OH.

172. The method of any one of claims 1-171, wherein the oligonucleotide is elongated under an inert atmosphere, preferably argon or nitrogen gas.

173. The method of any one of claims 1-172, wherein elongating the oligonucleotide is performed between 35 °C and 45 °C.

174. The method of any one of claims 1-173, wherein removing the 3′ blocking moiety is performed between 45 °C and 55 °C.

175. The method of any one of claims 1-174, wherein the elongated oligonucleotide without the 3′ blocking moiety is buffer exchanged and/or concentrated using a desalting chamber.

176. A composition comprising the oligonucleotide made according to the method of any one of claims 1-175.

177. The composition of claim 176, wherein the oligonucleotide is substantially free of depurination or depyrimidination impurities.

178. The composition of claim 176 or 177, wherein the oligonucleotide is substantially free of N-3- cyanoethylthymine (CNET) impurities.

179. The composition of any one of claims 176-178, wherein the oligonucleotide is substantially free of N(2)-actyl-2,6-diaminopurine and/or an isobutyryl diaminopurine impurities.

180. The composition of any one of claims 176-179, wherein the oligonucleotide is substantially free of methylcytosine.

181. The composition of any one of claims 176-180 is substantially free of Class IV impurities, preferably no detectable level of Class IV impurities.

182. A system for template-free synthesis of an oligonucleotide comprising:

a first reaction chamber comprising a transferase; and

a second reaction chamber comprising a hydrolase;

wherein the system is configured to flow an oligonucleotide in a solution from the first reaction chamber to the second reaction chamber while retaining the transferase in the first reaction chamber, and flow said oligonucleotide in the solution from the second chamber back to the first reaction chamber while retaining the hydrolase in the second reaction chamber.

183. The system of claim 182, further comprising a temperature regulator that controls a temperature of the solution in the system.

184. The system of claim 183, wherein the temperature regulator is configured to control the temperature of the solution in the first reaction chamber or the second reaction chamber.

185. The system of any one of claims 182-184, comprising one or more conduits that connects the first reaction chamber and the second reaction chamber.

186. The system of claim 185, comprising an in-line temperature regulator that controls a temperature of the solution in at least one of the one or more conduits.

187. The system of claim 185 or 186, wherein the one or more conduits comprises a first set of conduits connecting an outlet of the first reaction chamber to an inlet of the second reaction chamber, and a second set of conduits connecting an outlet of the second reaction chamber to an inlet of the first reaction chamber.

188. The system of any one of claims 182-187, comprising one or more pumps configured to flow the solution from the first reaction chamber to the second reaction chamber, and from the second reaction chamber to the first reaction chamber.

189. The system of claim 188, wherein the one or more pumps are configured to control a flow rate of the solution.

190. The system of any one of claims 182-189, further comprising a third reaction chamber comprising a second transferase, wherein the system is further configured to selectively flow said oligonucleotide in the solution from the second chamber to the third reaction chamber while retaining the hydrolase in the second reaction chamber, and flow the oligonucleotide in the solution from the third reaction chamber to the second reaction chamber while retaining the second transferase in the third reaction chamber.

191. The system of claim 190, wherein the transferase and the second transferase are different types of transferase.

192. The system of any one of claims 182-191, wherein the system further comprises one or more valves that selectively controls a flow pathway of the solution in the system.

193. The system of any one of claims 182-192, wherein the hydrolase can remove a 3′ blocking moiety from the oligonucleotide in the solution.

194. The system of any one of claims 182-193, wherein the hydrolase is a phosphatase, preferably an alkaline phosphatase.

195. The system of any one of claims 182-194, wherein the transferase can react a nucleotide triphosphate (NTP) comprising a 3′ blocking moiety, or an analog thereof comprising a 5′ phosphate analog, with the oligonucleotide to elongate the oligonucleotide.

196. The system of claim 195, wherein the 5′ phosphate analog is a 5′-(α-P-thio)phosphate moiety.

197. The system of claim 195 or 196, wherein the NTP comprising the 3′ blocking moiety or the analog thereof is a ribonucleotide or a deoxyribonucleotide, or further comprises a 2′ modification, preferably 2′-F or 2′-O-methyl.

198. The system of any one of claims 195-197, wherein the 3′ blocking moiety of the NTP or the analog thereof is a phosphate moiety.

199. The system of any one of claims 182-198, wherein the hydrolase can remove a 3′-blocking moiety from unreacted nucleotides comprising a 3′ blocking moiety in the solution.

200. The system of claim 199, wherein the unreacted nucleotides are ribonucleotides or deoxyribonucleotides, or comprise a 2′ modification, or preferably 2′-F or 2′-O-methyl.

201. The system of claim 199 or 200, wherein the 3′ blocking moiety of the unreacted nucleotides is a phosphate moiety or a 5′ phosphate analog.

202. The system of claim 201, wherein the 5′ phosphate analog of the unreacted nucleotides is a 5′-(α-P-thio)phosphate moiety.

203. The system of any one of claims 182-202, wherein the hydrolase can remove one or more 5′ phosphate moieties and/or 5′-(α-P-thio)phosphate moieties from the unreacted nucleotides in the solution.

204. The system of any one of claims 182-203, wherein the hydrolase is immobilized on a solid support.

205. The system of any one of claims 182-204, wherein the transferase comprises a polymerase from the DNA polymerase X family; a template independent transferase, preferably a terminal deoxynucleotidyl transferase (TdT); or an ssRNA ligase.

206. The system of any one of claims 182-205, wherein the transferase is immobilized on a solid support.

207. The system of any one of claims 182-206, wherein the first reaction chamber further comprises a pyrophosphatase, preferably an inorganic pyrophosphatase.

208. The system of any one of claims 182-207, further comprising a third reaction chamber comprising a pyrophosphatase, wherein the system is configured to flow the oligonucleotide in the solution from the first reaction chamber to the third reaction chamber comprising the pyrophosphatase while retaining the transferase in the first reaction chamber, and selectively flow said oligonucleotide in the solution from the third reaction chamber comprising the pyrophosphatase to the first reaction chamber comprising the transferase or the second reaction chamber comprising the hydrolase, wherein the pyrophosphate is retained in the third reaction chamber comprising the pyrophosphatase.

209. The system of claim 207 or 208, wherein the pyrophosphatase is immobilized on a solid support.

210. The system of claim 207, wherein the transferase and phosphatase are fused together.

211. The system of claim 207, wherein the transferase and phosphatase are immobilized on the same solid support or are immobilized on different solid supports.

212. The system of any one of claims 182-211, wherein the first reaction chamber or the second reaction chamber is a column.

213. The system of claim 212, wherein the first reaction chamber comprises a fixed bed comprising the transferase immobilized on a solid support; or a fluidized bed comprising the transferase immobilized on a solid support; or comprises a filter that prevents passage of the transferase and allows passage of the oligonucleotide.

214. The system of claim 212 or 213, wherein the second reaction chamber comprises a fixed bed comprising the hydrolase immobilized on a solid support or a fluidized bed comprising the hydrolase immobilized on a solid support or a filter that prevents passage of the hydrolase and allows passage of the oligonucleotide.

215. The system of any one of claims 182-211, wherein the first reaction chamber or the second reaction chamber is a batch reaction chamber, optionally wherein the batch reaction chamber comprises an impeller.

216. The system of claim 215, wherein:

the first reaction chamber comprises a rotating bed reactor comprising the transferase immobilized on a solid support; and/or

the second reaction chamber comprises a rotating bed reactor comprising the hydrolase immobilized on a solid support.

217. The system of any one of claims 182-216, further comprising a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from an oligonucleotide.

218. The system of claim 217, wherein the purification chamber comprises a column, preferably a liquid chromatography column, a size exclusion column, an ion exchange column, or a reverse phase column.

219. The system of claim 217, wherein the purification chamber is part of a tangential flow filtration system.

220. The system of any one of claims 182-219, wherein the first reaction chamber or the second reaction chamber are cleanable or replaceable.

221. The system of any one of claims 182-220, wherein:

the first reaction chamber comprises an inlet and an outlet;

the second reaction chamber comprises an inlet and an outlet; and

the system further comprising a reagent reservoir comprising an inlet and an outlet; and

wherein the system is configured to flow the oligonucleotide in the solution from an outlet of one of the reagent reservoir to the inlet of the first reaction chamber, from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from the outlet of the second reaction chamber to an inlet of the reagent reservoir.

222. The system of claim 221, wherein the reagent reservoir comprises an impeller.

223. The system of claim 221 or 222, wherein the reagent reservoir is replaceable or cleanable.

224. The system of any one of claims 221-223, further comprising a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the reagent reservoir.

225. The system of any one of claims 221-223, further comprising a product reservoir comprising an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution from an outlet of the second reaction chamber to an inlet of the product reservoir, from an outlet of the product reservoir to an inlet of the first reaction chamber.

226. The system of claim 224 or 225, wherein the product reservoir comprises an impeller.

227. The system of any one of claims 224-226, wherein the product reservoir is replaceable or cleanable.

228. The system of any one of claims 221-227, wherein the reagent reservoir comprises the oligonucleotide and a nucleotide comprising a 3′ blocking moiety.

229. The system of any one of claims 221-228, further comprising the purification chamber, wherein the purification chamber comprises an inlet and an outlet, wherein the system is configured to flow the oligonucleotide in the solution, in order, from the outlet of the second reaction chamber to the inlet of the purification chamber, and from the outlet of the purification chamber to the inlet of the reagent reservoir.

230. The system of any one of claims 221-229, wherein the system comprises the purification chamber, wherein the system is configured to flow the oligonucleotide, in the solution, from the outlet of the reagent reservoir to the inlet of the first reaction chamber, from the outlet of the first reaction chamber to the inlet of the second reaction chamber, from the outlet of the second reaction chamber to the inlet of the purification chamber, from the outlet of the purification chamber to the inlet of the product reservoir, and from the outlet of the product reservoir to the inlet of the reagent reservoir.

231. The system of any one of claims 182-220, wherein:

the first reaction comprises an inlet and an outlet;

the second reaction chamber comprises an inlet and an outlet; and

the system further comprising a reagent reservoir comprising an inlet and an outlet, and one or more diverter valves configured to control a flow pathway of the solution;

wherein the system is configured to controllably flow the oligonucleotide in the solution through the flow pathway selected from a plurality of flow pathways comprising (i) a first flow pathway comprising flow of the oligonucleotide in the solution from the outlet of the reagent reservoir to the inlet of the first reaction chamber without flowing through the second reaction chamber, and (ii) a second flow pathway comprising flow of the oligonucleotide in the solution from the outlet of the reagent reservoir to the inlet of the second reaction chamber without flowing through the first reaction chamber.

232. The system of claim 231, wherein the system is configured to automatically select the flow pathway.

233. The system of claim 231 or 232, wherein:

the first flow pathway comprises flow of the solution from the outlet of the first reaction chamber to the inlet of the reagent reservoir without flowing through the second reaction chamber;

and

the second flow pathway comprises flow of the solution from the outlet of the second reaction chamber to the inlet of the reagent reservoir without flowing through the first reaction chamber.

234. The system of any one of claims 231-233, wherein the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a third flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent reservoir to the inlet of the purification chamber without flowing through the first reaction chamber or the second reaction chamber.

235. The system of claim 234, wherein the third flow pathway further comprises flow of the solution from the outlet of the purification chamber to the inlet of the reagent reservoir without flowing through the first reaction chamber or the second reaction chamber.

236. The system of any one of claims 231-235, wherein the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a fourth flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent chamber to an inlet of the first reaction chamber, from an outlet of the first reaction chamber into an inlet of the purification chamber, and from an outlet of the purification chamber into an inlet of the reagent reservoir.

237. The system of any one of claims 231-236, wherein the system further comprises the purification chamber comprising an inlet and an outlet, and the plurality of flow pathways further comprises a fifth flow pathway comprising flow of the oligonucleotide in solution from the outlet of the reagent chamber to an inlet of the second reaction chamber, from an outlet of the second reaction chamber into an inlet of the purification chamber, and from an outlet of the purification chamber into an inlet of the reagent reservoir.

238. The system of any one of claims 231-237, wherein the reagent reservoir comprises an impeller.

239. The system of any one of claims 182-220, wherein

the transferase in the first reaction chamber is immobilized on a solid support, and the first reaction chamber comprises an inlet, an outlet, and an impeller configured to suspend the solid support comprising the transferase; and

and the hydrolase in the second reaction chamber is immobilized on a solid support, and the second reaction chamber comprises an inlet, an outlet, and an impeller configured to suspend the solid support comprising the hydrolase.

240. The system of any one of claims 182-220, wherein

the transferase in the first reaction chamber is immobilized on a solid support within a

rotating bed reactor, and the first reaction chamber comprises an inlet and an outlet; and

and the hydrolase in the second reaction chamber is immobilized on a solid support within a rotating bed reactor, and the second reaction chamber comprises an inlet and an outlet.

241. The system of claim 239 or 240, comprising one or more diverter valves configured to alternatively direct flow of the solution through (i) a first flow pathways comprising flow of the solution from the outlet of the first reaction chamber to an inlet of the purification chamber, and from an outlet of the purification chamber to the inlet of the first reaction chamber, or (ii) a second flow pathway comprising flow of the solution from the outlet of the first reaction chamber to the inlet of the second reaction chamber, and from an outlet of the second reaction chamber to the inlet of the first reaction chamber.

242. A system for template-free synthesis of an oligonucleotide comprising:

a reagent reservoir;

a first column configured to substantially separate a transferase from an oligonucleotide comprising a 3′ blocking moiety; and

a second column configured to substantially separate a hydrolase from an oligonucleotide without the 3′ blocking moiety;

wherein the system is configured to (i) flow a solution comprising an oligonucleotide comprising the 3′ blocking moiety and a transferase from the reagent reservoir to the first column, (ii) substantially separate the oligonucleotide comprising the 3′ blocking moiety from the transferase in the first column, (iii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety from the first column to the reagent reservoir, (iv) flow the solution comprising an oligonucleotide without the 3′ blocking moiety and a hydrolase to the second column, (v) substantially separate the oligonucleotide without the 3′ blocking moiety from the hydrolase in the second column, and (vi) flow the solution comprising the oligonucleotide without the 3′ blocking moiety from the second column to the reagent reservoir.

243. The system of claim 242, comprising a plurality of conduits that connects the first column, the second column, and the reagent reservoir.

244. The system of claim 242 or 243, further comprising a purification chamber configured to separate unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety.

245. The system of claim 244, further comprising one or more conduits that connects the purification chamber and the reagent reservoir.

246. The system of claim 244 or 245, wherein the purification chamber comprises a column, preferably a size exclusion column, an ion exchange column, or a reverse phase column, or wherein the purification chamber is part of a tangential flow filtration system.

247. The system of any one of claims 244-246, wherein the system is further configured to (vii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety from the reagent reservoir to the purification chamber, (viii) separate unreacted nucleotides or reaction byproducts in the solution from the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety in the purification chamber, and (ix) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety or the oligonucleotide without the 3′ blocking moiety from the purification chamber to the reagent reservoir.

248. The system of any one of claims 242-247, comprising one or more diverter valves configured to controllably flow the solution from the reagent reservoir to the first column or the second column.

249. The system of claim 248, wherein the one or more diverter valves is configured to select a flow path for the solution, wherein the flow path is selected from a plurality of flow paths comprising (i) flow of the solution from the reagent reservoir to the first column and from the first column to the reagent reservoir and (ii) flow of the solution from the reagent reservoir to the second column and from the second column to the reagent reservoir.

250. The system of any one of claims 244-249, wherein system comprises the purification chamber, and the one or more diverter valves is further configured to controllably flow the solution from the reagent reservoir to the purification chamber.

251. The system of claim 249 or 250, wherein the plurality of flow paths further comprises (iii) flow of the solution from the reagent reservoir to the purification chamber and from the purification chamber to the reagent reservoir.

252. The system of any one of claims 242-251, further comprising one or more wash buffer reservoirs connected to the first column, the second column, or the purification chamber.

253. The system of claim 252, wherein the one or more diverter valves is configured to controllably flow wash buffer from the wash buffer reservoir to the first column, the second column, or the purification chamber.

254. The system of claim 252 or 253, wherein the one or more diverter valves is configured to select a wash buffer flow path for wash buffer in the one or more wash buffer reservoirs, wherein the flow path is selected from a plurality of flow paths comprising (i) flow of the wash buffer from the one or more wash buffer reservoirs to the first column and from the first column to a system

waste outlet and (ii) flow of the wash buffer from the one or more wash buffer reservoirs to the second column and from the second column to the system waste outlet.

255. The system of claim 254, wherein the system comprises the purification chamber, and wherein the plurality of flow paths further comprises (iii) flow of the wash buffer from the one or more wash buffer reservoirs to the purification chamber and from the purification chamber to the system waste outlet.

256. The system of any one of claims 242-255, further comprising a temperature regulator that controls a temperature of the solution in the system.

257. The system of claim 256, wherein the temperature regulator is configured to control the temperature of the solution in the first column or the second column.

258. The system of claim 256 or 257, comprising an in-line temperature regulator that controls a temperature of the solution in one or more conduits of the system.

259. The system of any one of claims 242-258, comprising one or more pumps configured to flow the solution from the reagent reservoir to the first column or from the reagent reservoir to the second column.

260. The system of claim 259, wherein the system comprises the purification chamber, and the one or more pumps are further configured to flow the solution from the reagent reservoir to the purification chamber.

261. The system of claim 259 or 260, wherein the one or more pumps are configured to control a flow rate of the solution.

262. The system of any one of claims 242-261, wherein the first column is further configured to substantially separate a pyrophosphatase from the oligonucleotide comprising a 3′ blocking moiety.

263. The system of any one of claims 242-261, further comprising a third column configured to substantially separate a pyrophosphatase from the oligonucleotide comprising a 3′ blocking moiety, wherein the system is further configured to flow a solution comprising the oligonucleotide comprising the 3′ blocking moiety and the pyrophosphatase from the reagent reservoir to the third column, (ii) substantially separate the oligonucleotide comprising the 3′ blocking moiety from the pyrophosphatase in the third column, and (iii) flow the solution comprising the oligonucleotide comprising the 3′ blocking moiety from the third column to the reagent reservoir.

264. The system of any one of claims 242-263, wherein the first column comprises

a filter that prevents passage of the transferase and allows passage of the oligonucleotide; or

a resin that binds the transferase or

an ion exchange column, an affinity column, a size exclusion column, or reverse phase column.

265. The system of any one of claims 242-264, wherein the second column comprises

a filter that prevents passage of the hydrolase and allows passage of the oligonucleotide; or

a resin that binds the transferase; or

an ion exchange column, an affinity column, a size exclusion column, or reverse phase column.

266. The system of any one of claims 242-265, wherein the reagent reservoir comprises an impeller.

267. The system of any one of claims 242-266, wherein the reagent reservoir comprises a reagent port.

268. The system of any one of claims 242-267, wherein the first column or the second column are cleanable or replaceable.

269. The system of any one of claims 182-268, wherein the system comprises a degassing system or a sparging system.

270. The system of claim 269, wherein the sparging system is an in-line sparging system or is configured to sparge liquids in a reservoir, and/or is configured to sparge using an inert gas.

271. The system of claim 269 wherein the degasser system is configured to remove oxygen from the system.

272. The system of claim 183 or 256, wherein the temperature regulator is a jacketed stir tank.

273. The system of any one of claims 217-219, 229, 230, 234-237, 241, 244-255, and 260, wherein the purification chamber is configured to concentrate the elongated oligonucleotide comprising the 3′ blocking moiety.

274. The system of any one of claims 217-219, 229, 230, 234-237, 241, 244-255, 260, and 273, wherein the purification chamber comprises a membrane with a molecular weight cutoff of about 500 kDa to about 5000 kDa.