

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 September 2010 (30.09.2010)

PCT

(10) International Publication Number
WO 2010/111686 A2

(51) International Patent Classification:

C12Q 1/48 (2006.01) *G01N* 33/573 (2006.01)
C12N 9/12 (2006.01) *G01N* 33/58 (2006.01)

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(21) International Application Number:

PCT/US2010/028967

(22) International Filing Date:

26 March 2010 (26.03.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/164,324	27 March 2009 (27.03.2009)	US
61/184,770	5 June 2009 (05.06.2009)	US
61/242,771	15 September 2009 (15.09.2009)	US
61/245,457	24 September 2009 (24.09.2009)	US
61/263,974	24 November 2009 (24.11.2009)	US
61/289,388	22 December 2009 (22.12.2009)	US
61/293,616	8 January 2010 (08.01.2010)	US
61/293,618	8 January 2010 (08.01.2010)	US
61/299,919	29 January 2010 (29.01.2010)	US
61/299,917	29 January 2010 (29.01.2010)	US
61/307,356	23 February 2010 (23.02.2010)	US

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: LABELED ENZYME COMPOSITIONS, METHODS & SYSTEMS

(57) Abstract: Disclosed herein are conjugates comprising a biomolecule linked to a label that have biological activity and are useful in a wide variety of biological applications. For example, provided herein are labeled polymerase conjugates including a polymerase linked to one or more labels, wherein the conjugate has polymerase activity. Such conjugates can exhibit enhanced biological activity and/or superior detectability as compared to conventional labeled polymerases. Also disclosed herein are improved methods for preparing such conjugates, and methods and systems for using such conjugates in biological applications such as nucleotide incorporation, primer extension and single molecule sequencing.



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LABELED ENZYME COMPOSITIONS, METHODS & SYSTEMS

[0001] This application claims the filing date benefit of U.S. Provisional Application Nos.: 61/164,324, filed on March 27, 2009; 61/184,770, filed on June 5, 2009; 61/242,771, filed on September 15, 2009; 61/245,457, filed on September 24, 2009; 61/263,974, filed on November 24, 2009; 61/289,388; filed on December 22, 2009; 61/293,618, filed on January 8, 2010; 61/293,616, filed on January 8, 2010; 61/299,919, filed on January 29, 2010; 61/299,917, filed on January 29, 2010; 61/307,356, filed on February 23, 2010. The contents of each of the foregoing patent applications are incorporated by reference in their entirety.

FIELD

[0002] The present disclosure relates generally to conjugates comprising a biomolecule linked to a label, for use in a variety of biological applications. More particularly, disclosed herein are labeled polymerase conjugates comprising a polymerase linked to a label, wherein the conjugate has polymerase activity.

BACKGROUND

[0003] Labeling of biomolecules is frequently performed in biological assays. Such labeling studies have been widely used to elucidate structural and/or functional properties of various biomolecules, including carbohydrates, lipids, nucleic acids, nucleotides and proteins. Enzymes are of particular interest because they catalyze fundamental biochemical reactions within living organisms. For example, DNA and RNA polymerases assist in genomic replication and transcription by catalyzing the polymerization of nucleotides into nucleic acids.

[0004] Conventional labeling techniques generally involve the attachment of one or few organic labels comprising fluorescent small molecules, e.g., dyes, to the biomolecule of interest. However, such labeled conjugates are generally not suitable for use in single molecule assays due the toxicity effect of the label on the biomolecule, and/or the poor detectability (as characterized, for example, by low signal/noise ratio, brightness, e.g., quantum yield, signal lifetime, etc) and photostability of such conjugates. There is therefore a need in the art for labeled biomolecule conjugates that emit stronger and more stable signals than is feasible with conjugates produced by conventional labeling methods, and that retain sufficient biological activity for use in single molecule assays.

[0005] Disclosed herein are improved labeled biomolecule conjugates, as well as novel methods of making and using such conjugates. Such conjugates comprise labeled biomolecules exhibiting improved biological activity, detectability and/or photostability and that are suitable for use in single molecule assays. In some embodiments, the conjugates comprise a biomolecule linked to multiple dye labels that retain sufficient biological activity for use in single molecule assays.

[0006] The superior detectability of the conjugates of the present disclosure permits a wide range of powerful new approaches not hitherto feasible using conventional labeling methods, including, for example, extended imaging of biological samples over an extended period of time, real time *in situ* visualization of biomolecules or biomolecular activity *in vivo* or *in vitro*, optical coding of biomolecules, physical manipulation of biomolecules and/or biomolecular sorting, all of which can optionally be performed in high-throughput format.

[0007] For example, disclosed herein are labeled polymerase conjugates comprising a polymerase linked to a label that emit signals of superior intensities and durations, thus improving their performance in single molecule sequencing applications. In some embodiments, the labeled polymerase conjugates include multiple dyes (typically three or more) linked in tandem to a single polymerase without significant loss of polymerase activity. In other embodiments, the labeled polymerase conjugates comprise a nanoparticle label that typically emits stronger and more stable signals relative to conventional organic dyes.

[0008] The labeled polymerase conjugates provided herein can undergo FRET with an acceptor-labeled nucleotide bound to the active site in such a manner that the resulting FRET-based signal is readily detectable in a single molecule system, and also emit signals of sufficient duration to permit longer “reads” from a single nucleic acid molecule, thus permitting single molecule reads of increased length and accuracy. Such conjugates also retain high levels of polymerase activity, thus increasing the efficiency of single molecule sequencing systems using such conjugates.

[0009] The production of such improved conjugates is associated with several technical challenges. For example, biomolecules labeled with nanoparticles frequently exhibit a high degree of aggregation; it can also be difficult to precisely

control the ratios at which the biomolecule will attach to the nanoparticle, a problem compounded by the difficulty of determining the stoichiometric composition (i.e., ratio of biomolecule to nanoparticle) of the resulting conjugates. Similarly, while the detectability of conjugates comprising organic dye labels can be improved by increasing the number of dye labels linked to the biomolecule, such increased dye loading is typically accompanied by a reduction or loss in activity of the biomolecule. There remains a need in the art for labeled biomolecule conjugates exhibiting reduced aggregation and increased biomolecular activity along with superior detectability. There is also a need for improved methods for conjugating biomolecules, e.g., proteins, to labels wherein the stoichiometry of the conjugated components can be reliably controlled and the activity of the biomolecule preserved.

SUMMARY

[0010] Disclosed herein are labeled biomolecule conjugates useful in a wide range of biological applications, methods of making and using such conjugates, as well as systems, apparatuses and kits comprising such conjugates.

[0011] For example, in one embodiment, the disclosure relates to a labeled polymerase conjugate comprising a polymerase linked to a plurality of labels. The conjugate can have polymerase activity.

[0012] In some embodiments, the disclosure relates to a method for creating a labeled polymerase conjugate, comprising linking a polymerase to a plurality of labels to form a labeled polymerase conjugate having polymerase activity.

[0013] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising a polymerase linked to a plurality of labels to form a labeled polymerase conjugate, where the conjugate has polymerase activity, and where at least one label of the conjugate performs energy transfer with a labeled nucleotide bound to an active site of the polymerase.

[0014] In some embodiments, the disclosure relates to a method for generating a signal, comprising: contacting a labeled polymerase conjugate including a polymerase linked to a label with a labeled nucleotide under conditions where the polymerase catalyzes the incorporation of the labeled nucleotide into a nucleic acid, and the label emits, or induces the emission of, a signal indicative of such nucleotide incorporation.

[0015] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising: a polymerase linked to one or more labels, where the conjugate has polymerase activity and emits upon continuous excitation a total photon count of at least 10^8 photons before irreversibly photobleaching. In some embodiments, the conjugate emits a total photon count of at least 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 or 10^9 photons as measured using a test detection system.

[0016] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising: a first member of a binding pair linked to a polymerase; and a second member of the binding pair linked to at least one label, where the first and the second member of the binding pair are linked to each other to form a labeled polymerase conjugate having polymerase activity.

DETAILED DESCRIPTION OF THE DRAWINGS

[0017] The following figures form part of the present specification and are included to further demonstrate certain aspects of the disclosure herein by way of illustrating non-limiting embodiments and examples. This disclosure may be better understood by reference to one or more of these figures in combination with the detailed description of specific embodiments presented herein.

[0018] FIG. 1 depicts an exemplary reaction pathway for preparing a dye-labeled polymerase conjugate according to the present disclosure.

[0019] FIG. 2 depicts an exemplary labeled polymerase conjugate comprising a polymerase linked to multiple Cy3 dyes according to the present disclosure

[0020] FIG. 3 depicts one exemplary method for preparing a dye-labeled polymerase conjugate according to the present disclosure.

[0021] FIG. 4 depicts the results of a nucleotide binding assay using a labeled polymerase conjugate according to the methods described in Example 2.

[0022] FIG. 5 depicts the results of a nucleic acid binding assay using a labeled polymerase conjugate according to the methods described in Example 3.

[0023] FIG. 6 depicts the results of a primer extension assays using labeled polymerase conjugates according to the methods described in Examples 4 and 5.

[0024] FIG. 7 depicts depicts the results of a nucleic acid binding assay using a labeled polymerase conjugate according to the methods described in Example 4 and 5.

[0025] FIG. 8 depicts the dye loading distribution, as measured by UV absorbance, of an exemplary labeled polymerase conjugate.

[0026] FIG. 9 depicts the detection apparatus used to monitor nucleotide incorporation using an exemplary labeled polymerase conjugate.

[0027] FIG. 10 depicts a graph showing the average donor lifetimes observed using an exemplary labeled polymerase conjugate.

[0028] FIG. 11 depicts a graph showing the FRET efficiencies observed using an exemplary labeled polymerase conjugate.

[0029] FIG. 12 depicts exemplary fluorescence traces observed during a single molecule nucleotide incorporation assay using an exemplary labeled polymerase conjugate.

[0030] FIG. 13 depicts exemplary fluorescence traces observed during a single molecule nucleotide incorporation assay using an exemplary labeled polymerase conjugate.

[0031] FIG. 14 depicts sequence data obtained using exemplary labeled polymerase conjugates according to the methods described in Example 6.

[0032] FIG. 15 depicts fluorescence data obtained during a nucleotide incorporation reaction using exemplary labeled polymerase conjugates according to the methods described in Example 6.

[0033] FIG. 16 depicts the results of an assay for polymerase activity using a primer extension assay.

[0034] FIG. 17 depicts an exemplary reaction pathway for forming a labeled polymerase conjugate according to the present disclosure.

[0035] FIG. 18 depicts a second exemplary reaction pathway for forming a labeled polymerase conjugate according to the present disclosure.

[0036] FIG. 19 depicts a third exemplary reaction pathway for forming a labeled polymerase conjugate according to the present disclosure.

[0037] FIG. 20 depicts a fourth exemplary reaction pathway for forming a labeled polymerase conjugate according to the present disclosure.

[0038] FIG. 21 depicts the structure of an exemplary nucleotide that can be used in conjunction with the labeled polymerase conjugates of the present disclosure according to the methods provided herein.

DETAILED DESCRIPTION OF THE SEQUENCE LISTINGS

[0039] SEQ ID NO: 1 comprises the amino acid sequence of an *E. coli* K12 DNA polymerase.

[0040] SEQ ID NO: 2 comprises the amino acid sequence of the Klenow form of an *E. coli* K12 DNA polymerase.

[0041] SEQ ID NO: 3 comprises the amino acid sequence of a DNA polymerase of the bacteriophage Phi-29.

[0042] SEQ ID NO: 4 comprises the amino acid sequence of a peptide linker, herein referred to as "H-linker".

[0043] SEQ ID NO: 5 comprises the amino acid sequence of a peptide linker, herein referred to as "F-linker".

[0044] SEQ ID NO: 6 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a polycysteine tag and the F-linker sequence at its N-terminus.

[0045] SEQ ID NO: 7 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a polylysine tag and the F-linker sequence at its N-terminus.

[0046] SEQ ID NO: 8 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a His-tag, an F-linker peptide and a transglutaminase tag at its N-terminus.

[0047] SEQ ID NO: 9 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a protein kinase A (PKA) tag and the F-linker at its N-terminus.

[0048] SEQ ID NO: 10 comprises the amino acid sequence of a biotin acceptor peptide in an exemplary biotin ligase recognition sequence.

[0049] SEQ ID NO: 11 comprises the amino acid sequence of HBP1, a Phi-29 polymerase peptide comprising a His-tag and biotin acceptor peptide sequence at its N-terminus.

[0050] SEQ ID NO: 12 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a His-tag and the H-linker at its N-terminus.

[0051] SEQ ID NO: 13 comprises the amino acid sequence of a Phi-29 polymerase peptide comprising a His-tag and the F-linker at its N-terminus.

[0052] SEQ ID NO: 14 comprises the amino acid sequence of HP1, a Phi-29 polymerase peptide that lacks exonuclease activity and comprises an N-terminal His-tag, an intervening linker sequence, and the D12A and D66A mutations.

[0053] SEQ ID NO: 15 comprises the amino acid sequence of a Cyanophage S-CBP1 DNA polymerase.

[0054] SEQ ID NO: 16 comprises the amino acid sequence of a Cyanophage S-CBP2 DNA polymerase.

[0055] SEQ ID NO: 17 comprises the amino acid sequence of a Cyanophage S-CBP3 DNA polymerase.

[0056] SEQ ID NO: 18 comprises the amino acid sequence of a Cyanophage Syn-5 DNA polymerase.

[0057] SEQ ID NO: 19 comprises the amino acid sequence of a Cyanophage S-CBP42 DNA polymerase.

[0058] SEQ ID NO: 20 comprises the amino acid sequence of be a Synechococcus phage P60 DNA polymerase.

[0059] SEQ ID NO: 21 comprises the amino acid sequence of a Roseobacter phage SIO1 DNA polymerase.

[0060] SEQ ID NO: 22 comprises the amino acid sequence of a Oedogonium cardiacum chloroplast DNA Polymerase.

[0061] SEQ ID NO: 23 comprises the amino acid sequence of a Salterprovirus His1 polymerase.

[0062] SEQ ID NO: 24 comprises the amino acid sequence of a Salterprovirus His2 polymerase.

[0063] SEQ ID NO: 25 comprises the amino acid sequence of an Ostreococcus tauri V5 DNA polymerase.

[0064] SEQ ID NO: 26 comprises the amino acid sequence of an Ectocarpus siliculosus virus 1 DNA polymerase.

[0065] SEQ ID NO: 27 comprises the amino acid sequence of HP1 Q380A, a mutant form of HP1 comprising the mutation Q380A.

[0066] SEQ ID NO: 28 comprises the amino acid sequence of HP1 S388G, a mutant form of HP1 comprising the mutation S388G.

[0067] SEQ ID NO: 29 comprises the amino acid sequence of an RB69 polymerase comprising a His-tag at its N-terminus.

[0068] SEQ ID NO: 30 comprises the amino acid sequence of a GA-1 polymerase comprising a His-tag at its N-terminus.

[0069] SEQ ID NO: 31 comprises the amino acid sequence of a B103 polymerase comprising a His-tag at its N-terminus.

[0070] SEQ ID NO: 32 comprises the amino acid sequence of B103 polymerase.

[0071] SEQ ID NO: 33 comprises the amino acid sequence of a mutant B103 polymerase.

[0072] SEQ ID NO: 34 comprises the amino acid sequence of a second mutant B103 polymerase.

[0073] SEQ ID NO: 35 comprises the amino acid sequence of an M2Y DNA polymerase.

[0074] SEQ ID NO: 36 comprises the amino acid sequence of an Nf DNA polymerase.

[0075] SEQ ID NO: 37 comprises the amino acid sequence of an exemplary recognition sequence for the Tobacco Etch Virus (TEV) protease.

[0076] SEQ ID NO: 38 comprises the amino acid sequence of a Phi-29 polymerase fused to a TEV protease cleavage site.

[0077] SEQ ID NO: 39 comprises the amino acid sequence of a B103 polymerase fused to a TEV protease cleavage site.

[0078] SEQ ID NO: 40 comprises the amino acid sequence of a mutant B103 polymerase fused to a His-tag and biotin acceptor peptide sequence at its N-terminus.

[0079] SEQ ID NO: 41 comprises the nucleotide sequence of an oligonucleotide template used in a nucleotide incorporation assay.

[0080] SEQ ID NO: 42 comprises the nucleotide sequence of an oligonucleotide primer used in a nucleotide incorporation assay.

[0081] SEQ ID NO: 43 comprises the nucleotide sequence of a fluorescein-labeled oligonucleotide primer used to measure primer extension activity of a polymerase sample according to the exemplary assays as described herein.

[0082] SEQ ID NO: 44 comprises the nucleotide sequence of an exemplary polynucleotide template used in a stopped-flow assay for nucleotide incorporation kinetics as described, for example, in Example 10.

[0083] SEQ ID NO: 45 comprises the nucleotide sequence of an exemplary oligonucleotide primer used in a stopped-flow assay for nucleotide incorporation kinetics as described, for example, in Example 10.

[0084] SEQ ID NO: 46 comprises the nucleotide sequence of an exemplary polynucleotide template used in a stopped-flow assay for nucleotide incorporation kinetics as described, for example, in Example 10.

[0085] SEQ ID NO: 47 comprises the nucleotide sequence of an exemplary oligonucleotide primer used in a stopped-flow assay for nucleotide incorporation kinetics as described, for example, in Example 10.

[0086] SEQ ID NO: 48 comprises the nucleotide sequence of an exemplary polynucleotide hairpin template used in an exemplary assay for nucleotide incorporation as described, for example, in Example 8.

[0087] SEQ ID NO: 49 comprises the nucleotide sequence of an exemplary polynucleotide template used in an exemplary assay for nucleotide incorporation.

[0088] SEQ ID NO: 50 comprises the nucleotide sequence of an exemplary oligonucleotide primer used in an exemplary assay for nucleotide incorporation.

[0089] SEQ ID NO: 51 comprises the nucleotide sequence of an exemplary oligonucleotide primer used in an exemplary assay for nucleotide incorporation.

DETAILED DESCRIPTION

[0090] The present disclosure relates to compositions, methods, systems, apparatuses and kits comprising labeled biomolecule conjugates including a biomolecule linked to a label, wherein the conjugate has a biological activity that is characteristic of the biomolecule. Typically, the label of the conjugate emits, or is capable of emitting, a signal. In some embodiments, the label induces emission, or is capable of inducing emission (e.g., via energy transfer) of the signal. Optionally, the signal can indicate various aspects of the biological activity of the conjugate. In some embodiments, the conjugate can be visualized and tracked in real time, optionally in single molecule format. Also disclosed herein are improved methods

for preparing such conjugates, as well as methods, systems, apparatuses and kits for using such conjugates in biological applications, including for example single molecule reactions.

[0091] In some embodiments, the biomolecule is a polymerase and the labeled biomolecule conjugate is a labeled polymerase conjugate including a polymerase linked to a label, wherein the conjugate has polymerase activity.

[0092] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which these inventions belong. All patents, patent applications, published applications, treatises and other publications referred to herein, both *supra* and *infra*, are incorporated by reference in their entirety. If a definition and/or description is set forth herein that is contrary to or otherwise inconsistent with any definition set forth in the patents, patent applications, published applications, and other publications that are herein incorporated by reference, the definition and/or description set forth herein prevails over the definition that is incorporated by reference.

[0093] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA techniques, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook, J., and Russell, D.W., 2001, *Molecular Cloning: A Laboratory Manual*, Third Edition; Ausubel, F.M., et al., eds., 2002, *Short Protocols In Molecular Biology*, Fifth Edition.

[0094] As used herein, the terms “link”, “linked”, “linkage” and variants thereof comprise any type of fusion, bond, adherence or association that is of sufficient stability to withstand use in the particular biological application of interest. Such linkage can comprise, for example, covalent, ionic, hydrogen, dipole-dipole, hydrophilic, hydrophobic, or affinity bonding, bonds or associations involving van der Waals forces, mechanical bonding, and the like. Optionally, such linkage can occur between a combination of different molecules, including but not limited to: between a nanoparticle and a protein; between a protein and a label; between a linker and a functionalized nanoparticle; between a linker and a protein; and the like. Some examples of linkages can be found, for example, in Hermanson, G., *Bioconjugate Techniques*, Second Edition (2008); Aslam, M., Dent, A., *Bioconjugation: Protein*

Coupling Techniques for the Biomedical Sciences, London: Macmillan (1998); Aslam, M., Dent, A., *Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences*, London: Macmillan (1998).

[0095] As used herein, the term “linker” and its variants comprises any composition, including any molecular complex or molecular assembly, that serves to link two or more compounds.

[0096] As used herein, the term “polymerase” and its variants comprise any enzyme that can catalyze the polymerization of nucleotides (including analogs thereof) into a nucleic acid strand. Typically but not necessarily such nucleotide polymerization can occur in a template-dependent fashion. Such polymerases can include without limitation naturally occurring polymerases and any subunits and truncations thereof, mutant polymerases, variant polymerases, recombinant, fusion or otherwise engineered polymerases, chemically modified polymerases, synthetic molecules or assemblies, and any analogs, derivatives or fragments thereof that retain the ability to catalyze such polymerization. Optionally, the polymerase can be a mutant polymerase comprising one or more mutations involving the replacement of one or more amino acids with other amino acids, the insertion or deletion of one or more amino acids from the polymerase, or the linkage of parts of two or more polymerases. Typically, the polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. Some exemplary polymerases include without limitation DNA polymerases (such as for example Phi-29 DNA polymerase, reverse transcriptases and *E. coli* DNA polymerase) and RNA polymerases. The term “polymerase” and its variants, as used herein, also refers to fusion proteins comprising at least two portions linked to each other, where the first portion comprises a peptide that can catalyze the polymerization of nucleotides into a nucleic acid strand and is linked to a second portion that comprises a second polypeptide, such as, for example, a reporter enzyme or a processivity-enhancing domain. One exemplary embodiment of such a polymerase is Phusion® DNA polymerase (New England Biolabs), which comprises a *Pyrococcus*-like polymerase fused to a processivity-enhancing domain as described, for example, in U.S. Patent No. 6,627,424.

[0097] As used herein, the term “polymerase activity” and its variants, when used in reference to a given polymerase, comprises any in vivo or in vitro enzymatic activity characteristic of a given polymerase that relates to catalyzing the polymerization of nucleotides into a nucleic acid strand, e.g., primer extension activity, and the like. Typically, but not necessarily such nucleotide polymerization occurs in a template-dependent fashion. In addition to such polymerase activity, the polymerase can typically possess other enzymatic activities, for example, 3' to 5' exonuclease activity.

[0098] As used herein, the term “nucleotide” and its variants comprises any compound that can bind selectively to, or can be polymerized by, a polymerase. Typically, but not necessarily, selective binding of the nucleotide to the polymerase is followed by polymerization of the nucleotide into a nucleic acid strand by the polymerase; occasionally however the nucleotide may dissociate from the polymerase without becoming incorporated into the nucleic acid strand, an event referred to herein as a “non-productive” event. Such nucleotides include not only naturally occurring nucleotides but also any analogs, regardless of their structure, that can bind selectively to, or can be polymerized by, a polymerase. While naturally occurring nucleotides typically comprise base, sugar and phosphate moieties, the nucleotides of the present disclosure can include compounds lacking any one, some or all of such moieties. In some embodiments, the nucleotide can optionally include a chain of phosphorus atoms comprising three, four, five, six, seven, eight, nine, ten or more phosphorus atoms. In some embodiments, the phosphorus chain can be attached to any carbon of a sugar ring, such as the 5' carbon. The phosphorus chain can be linked to the sugar with an intervening O or S. In one embodiment, one or more phosphorus atoms in the chain can be part of a phosphate group having P and O. In another embodiment, the phosphorus atoms in the chain can be linked together with intervening O, NH, S, methylene, substituted methylene, ethylene, substituted ethylene, CNH_2 , $\text{C}(\text{O})$, $\text{C}(\text{CH}_2)$, CH_2CH_2 , or $\text{C}(\text{OH})\text{CH}_2\text{R}$ (where R can be a 4-pyridine or 1-imidazole). In one embodiment, the phosphorus atoms in the chain can have side groups having O, BH_3 , or S. In the phosphorus chain, a phosphorus atom with a side group other than O can be a substituted phosphate group. In the phosphorus chain, phosphorus atoms with an intervening atom other than O can be a

substituted phosphate group. Some examples of nucleotide analogs are described in Xu, U.S. Patent No. 7,405,281. In some embodiments, the nucleotide comprises a label and referred to herein as a “labeled nucleotide”; the label of the labeled nucleotide is referred to herein as a “nucleotide label”. In some embodiments, the label can be in the form of a fluorescent dye attached to the terminal phosphate group, i.e., the phosphate group most distal from the sugar. Some examples of nucleotides that can be used in the disclosed methods and compositions include, but are not limited to, ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, ribonucleotide polyphosphates, deoxyribonucleotide polyphosphates, modified ribonucleotide polyphosphates, modified deoxyribonucleotide polyphosphates, peptide nucleotides, modified peptide nucleotides, metallonucleosides, phosphonate nucleosides, and modified phosphate-sugar backbone nucleotides, analogs, derivatives, or variants of the foregoing compounds, and the like. In some embodiments, the nucleotide can comprise non-oxygen moieties such as, for example, thio- or borano- moieties, in place of the oxygen moiety bridging the alpha phosphate and the sugar of the nucleotide, or the alpha and beta phosphates of the nucleotide, or the beta and gamma phosphates of the nucleotide, or between any other two phosphates of the nucleotide, or any combination thereof.

[0099] As used herein, the term “nucleotide incorporation” and its variants comprises polymerization of one or more nucleotides into a nucleic acid strand.

[00100] As used herein, the term “biomolecule” and its variants comprises any compound isolated from a living organism, as well as analogs (including engineered and/or synthetic analogs), derivatives, mutants or variants and/or biologically active fragments of the same. For example, the biomolecule can be a protein (e.g., enzyme), nucleic acid, nucleotide, carbohydrate or lipid. In some embodiments, the biomolecule can be an engineered or synthetic analog of a compound isolated from a living cell that is structurally different from the compound but retains a biological activity characteristic of that compound. As used herein, the term “target” and its variants comprises any compound that is capable of binding specifically to a particular biomolecule. In one exemplary embodiment, the target of an enzyme can be, for example, a substrate of the enzyme.

[00101] As used herein, the term “biological activity” and its variants, when used in reference to a biomolecule (such as, for example, an enzyme) refers to any *in vivo* or *in vitro* activity that is characteristic of the biomolecule itself, including the interaction of the biomolecule with one or more targets. For example, biological activity can optionally include the selective binding of an antibody to an antigen, the enzymatic activity of an enzyme, and the like. Such activity can also include, without limitation, binding, fusion, bond formation, association, approach, catalysis or chemical reaction, optionally with another biomolecule or with a target molecule.

[00102] As used herein, the term “biologically active fragment” and its variants refers to any fragment, derivative or analog of a biomolecule that possesses an *in vivo* or *in vitro* activity that is characteristic of the biomolecule itself. For example, the biomolecule can be an antibody that is characterized by antigen-binding activity, or an enzyme characterized by the ability to catalyze a particular biochemical reaction, etc. Biologically active fragments can optionally exist *in vivo*, such as, for example, fragments which arise from post transcriptional processing or which arise from translation of alternatively spliced RNAs, or alternatively can be created through engineering, bulk synthesis, or other suitable manipulation. Biologically active fragments include fragments expressed in native or endogenous cells as well as those made in expression systems such as, for example, in bacterial, yeast, insect or mammalian cells. Because biomolecules often exhibit a range of physiological properties and because such properties can be attributable to different portions of the biomolecule, a useful biologically active fragment can be a fragment of a biomolecule that exhibits a biological activity in any biological assay. In some embodiments, the fragment or analog possesses 10%, 40%, 60%, 70%, 80% or 90% or greater of the activity of the biomolecule in any *in vivo* or *in vitro* assay of interest.

[00103] The term “modification” or “modified” and their variants, as used herein with reference to a protein, comprise any change in the structural, biological and/or chemical properties of the protein, particularly a change in the amino acid sequence of the protein. In some embodiments, the modification can comprise one or more amino acid mutations, including without limitation amino acid additions, deletions and substitutions (including both conservative and non-conservative substitutions).

[00104] As used herein, the terms “identical” or “percent identity,” and their variants, when used in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using any one or more of the following sequence comparison algorithms: Needleman-Wunsch (see, e.g., Needleman, Saul B.; and Wunsch, Christian D. (1970). *"A general method applicable to the search for similarities in the amino acid sequence of two proteins"* *Journal of Molecular Biology* 48 (3):443–53); Smith-Waterman (see, e.g., Smith, Temple F.; and Waterman, Michael S., “*Identification of Common Molecular Subsequences*” (1981) *Journal of Molecular Biology* 147:195–197); or BLAST (Basic Local Alignment Search Tool; see, e.g., Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, *"Basic local alignment search tool"* (1990) *J Mol Biol* 215 (3):403–410).

[00105] The terms “resonance energy transfer” and “RET” and their variants, as used herein, refer to a radiationless transmission of excitation energy from a first moiety, termed a donor moiety, to a second moiety termed an acceptor moiety. One type of RET includes Forster Resonance Energy Transfer (FRET), in which a fluorophore (the donor) in an excited state transfers its energy to a proximal molecule (the acceptor) by nonradiative dipole-dipole interaction. See, e.g., Forster, T. “Intermolecular Energy Migration and Fluorescence”, *Ann. Phys.*, 2:55-75, 1948; Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, 2nd ed. Plenum, New York. 367–394., 1999. RET also comprises luminescence resonance energy transfer, bioluminescence resonance energy transfer, chemiluminescence resonance energy transfer, and similar types of energy transfer not strictly following the Forster's theory, such as nonoverlapping energy transfer occurring when nonoverlapping acceptors are utilized. See, for example, *Anal. Chem.* 2005, 77: 1483-1487.

[00106] The term “conservative” and its variants, as used herein with reference to any change in amino acid sequence, refers to an amino acid mutation wherein one or more amino acids is substituted by another amino acid having highly similar properties. For example, one or more amino acids comprising nonpolar or aliphatic side chains (for example, glycine, alanine, valine, leucine, isoleucine or proline) can be substituted for each other. Similarly, one or more amino acids comprising polar,

uncharged side chains (for example, serine, threonine, cysteine, methionine, asparagine or glutamine) can be substituted for each other. Similarly, one or more amino acids comprising aromatic side chains (for example, phenylalanine, tyrosine or tryptophan) can be substituted for each other. Similarly, one or more amino acids comprising positively charged side chains (for example, lysine, arginine or histidine) can be substituted for each other. Similarly, one or more amino acids comprising negatively charged side chains (for example, aspartic acid or glutamic acid) can be substituted for each other. In some embodiments, the modified polymerase is a variant that comprises one or more of these conservative amino acid substitutions, or any combination thereof. In some embodiments, conservative substitutions for leucine include: alanine, isoleucine, valine, phenylalanine, tryptophan, methionine, and cysteine. In other embodiments, conservative substitutions for asparagine include: arginine, lysine, aspartate, glutamate, and glutamine.

[00107] The term “primer extension activity” and its variants, as used herein, when used in reference to a given polymerase, comprises any *in vivo* or *in vitro* enzymatic activity characteristic of a given polymerase that relates to catalyzing nucleotide incorporation onto the terminal 3'OH end of an extending nucleic acid molecule. Typically but not necessarily such nucleotide incorporation occurs in a template-dependent fashion. The primer extension activity is typically quantified as the total number of nucleotides incorporated (as measured by, e.g. radiometric or other suitable assay) by a unit amount of polymerase (in moles) per unit time (seconds) under a particular set of reaction conditions.

[00108] The terms “His tag” or “His-tag” and their variants as used herein refers to a stretch of amino acids comprising multiple histidine residues. Typically, the His tag can bind to metal ions, for example, Zn^{2+} , Ni^{2+} , Co^{2+} , or Cu^{2+} ions. Optionally, the His tag comprises 2, 3, 4, 5, 6, 7, 8 or more histidine residues. In some embodiments, the His tag is fused to the N- or C-terminus of a protein; alternatively, it can be fused at any suitable location within the protein.

[00109] As used herein, the term “binding pair” and its variants refers to two molecules, or portions thereof, which have a specific binding affinity for one another and typically will bind to each other in preference to binding to other molecules. Typically but not necessarily some or all of the structure of one member of a specific

binding pair is complementary to some or all of the structure possessed by the other member, with the two members being able to bind together specifically by way of a bond between the complementary structures, optionally by virtue of multiple noncovalent attractions. The two members of a binding pair are referred to herein as the “first member” and the “second member” respectively.

[00110] The following may be mentioned as non-limiting examples of molecules that can function as a member of a specific binding pair, without this being understood as any restriction: thyroxin-binding globulin, steroid-binding proteins, antibodies, antigens, haptens, enzymes, lectins, nucleic acids, repressors, oligonucleotides, polynucleotides, protein A, protein G, avidin, streptavidin, biotin, complement component C1q, nucleic acid-binding proteins, receptors, carbohydrates, complementary nucleic acid sequences, and the like. Examples of specific binding pairs include without limitation: an avidin moiety and a biotin moiety; an antigenic epitope and an antibody or immunologically reactive fragment thereof; an antibody and a hapten; a digoxigen moiety and an anti-digoxigen antibody; a fluorescein moiety and an anti-fluorescein antibody; an operator and a repressor; a nuclease and a nucleotide; a lectin and a polysaccharide; a steroid and a steroid-binding protein; an active compound and an active compound receptor; a hormone and a hormone receptor; an enzyme and a substrate; an immunoglobulin and protein A; and an oligonucleotide or polynucleotide and its corresponding complement.

[00111] As used herein, the term “biotin moiety” and its variants comprises biotin (cis-hexahydro-2-oxo-1H-thieno[3,4]imidazole-4-pentanoic acid) and any derivatives and analogs thereof, including biotin-like compounds. Such compounds include, for example, biotin-ε-N-lysine, biocytin hydrazide, amino or sulfhydryl derivatives of 2-iminobiotin and biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, sulfosuccinimideiminobiotin, biotinbromoacetylhydrazide, p-diazobenzoyl biocytin, 3-(N-maleimidopropionyl)biocytin, and the like. “Biotin moiety” also comprises biotin variants that can specifically bind to an avidin moiety.

[00112] The term “biotinylated” and its variants, as used herein, refer to any covalent or non-covalent adduct of biotin with other moieties such as biomolecules, e.g., proteins, nucleic acids (including DNA, RNA, DNA/RNA chimeric molecules,

nucleic acid analogs and peptide nucleic acids), proteins (including enzymes, peptides and antibodies), carbohydrates, lipids, etc.

[00113] The terms “avidin” and “avidin moiety” and their variants, as used herein, comprises the native egg-white glycoprotein avidin, as well as any derivatives, analogs and other non-native forms of avidin, that can specifically bind to biotin moieties. In some embodiments, the avidin moiety can comprise deglycosylated forms of avidin, bacterial streptavidins produced by selected strains of *Streptomyces*, e.g., *Streptomyces avidinii*, to truncated streptavidins, and to recombinant avidin and streptavidin as well as to derivatives of native, deglycosylated and recombinant avidin and of native, recombinant and truncated streptavidin, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercial products ExtrAvidin®, Captavidin®, Neutravidin® and Neutralite Avidin®. All forms of avidin-type molecules, including both native and recombinant avidin and streptavidin as well as derivatized molecules, e.g. nonglycosylated avidins, N-acyl avidins and truncated streptavidins, are encompassed within the terms “avidin” and “avidin moiety”. Typically, but not necessarily, avidin exists as a tetrameric protein, wherein each of the four tetramers is capable of binding at least one biotin moiety.

[00114] As used herein, the term “biotin-avidin bond” and its variants refers to a specific linkage formed between a biotin moiety and an avidin moiety. Typically, a biotin moiety can bind with high affinity to an avidin moiety, with a dissociation constant K_d typically in the order of 10^{-14} to 10^{-15} mol/L. Typically, such binding occurs via non-covalent interactions.

[00115] As used herein, the term “modification enzyme recognition site” refers to an amino acid recognition sequence that is chemically modified in an enzyme-catalyzed reaction, wherein the enzyme catalyzing the reaction exhibits specificity for the amino acid recognition sequence. The amino acid recognition sequence may be inserted into a protein of interest, for example by conventional recombinant DNA techniques. Examples of modification enzyme recognition sites include, but are not limited to a biotin ligase modification site, for example a site comprising the amino acid sequence GLNDIFEAQKIEWHE, for introducing a biotin moiety; a protein kinase modification site, for example a site comprising the amino acid sequence LRRASLG, for introducing a phosphorothioate moiety; and a transglutaminase modification site,

for example a site comprising the amino acid sequence PKPQQF, for introducing an amine moiety.

[00116] The terms “reporter” and “reporter moiety” and their variants, as used herein, refer to any moiety that generates, or causes to be generated, a detectable signal. Any suitable reporter moiety may be used, including luminescent, photoluminescent, electroluminescent, bioluminescent, chemiluminescent, fluorescent, phosphorescent, chromophore, radioisotope, electrochemical, mass spectrometry, Raman, hapten, affinity tag, atom, or an enzyme. The reporter moiety generates a detectable signal resulting from a chemical or physical change (e.g., heat, light, electrical, pH, salt concentration, enzymatic activity, or proximity events). A proximity event includes two reporter moieties approaching each other, or associating with each other, or binding each other. The appropriate procedures for detecting a signal, or change in the signal, generated by the reporter moiety are well known in the art. The reporter moieties can be linked to a solid surface, polymerase, nucleotide (or analog thereof), target nucleic acid molecule, or primer. In one embodiment, a nucleotide can be linked to a reporter moiety. The reporter moiety can generate a signal, or a change in a signal, upon excitation from an appropriated energy source (e.g., electromagnetic source). In another embodiment, the polymerase can be linked to a reporter moiety (e.g., energy transfer donor moiety), and the nucleotide (or analog thereof) can be linked to a reporter moiety (e.g., energy transfer acceptor moiety). The reporter moieties (energy transfer donor and acceptor moieties) can generate a signal, or a change in a signal, upon excitation from an appropriated energy source (e.g., electromagnetic source) and when the nucleotide is proximal to the polymerase. The nucleotide can be proximal to the polymerase when the nucleotide binds the polymerase or when the polymerase incorporates the nucleotide. Some energy transfer reporter moieties can be optically or spectrally detectable.

[00117] The term “label” and its variants, as used herein, comprises any optically detectable moiety and includes any moiety that can be detected using, for example, fluorescence, luminescence and/or phosphorescence spectroscopy, Raman scattering, or diffraction. Exemplary labels according to the present disclosure include fluorescent and luminescent moieties as well as quenchers thereof. Some typical labels include without limitation nanoparticles and organic dyes.

[00118] The term “attachment site” and its variants, as used herein, refer to any location or region on the biomolecule or the label that is capable of supporting attachment to another moiety. For example, the biomolecule can comprise one or more attachment sites for a label; alternatively the label (e.g., nanoparticle or organic dye moiety) can comprise one or more attachment sites for the biomolecule. The attachment site can variously comprise one or more functional groups (e.g., carboxyl, amine, thiol groups, etc), a surface ligand, one or more amino acid side chains, an exposed region of the metal surface, a bound metal ion, or any other suitable moiety capable of supporting attachment to, e.g., a biomolecule or label.

[00119] “Nanoparticle” may refer to any particle with at least one major dimension in the nanosize range. In general, nanoparticles can be made from any suitable metal (e.g., noble metals, semiconductors, etc.) and/or non-metal atoms. Nanoparticles can have different shapes, each of which can have distinctive properties including spatial distribution of the surface charge; orientation dependence of polarization of the incident light wave; and spatial extent of the electric field. The shapes include, but are not limited to: spheres, rods, discs, triangles, nanorings, nanoshells, tetrapods, nanowires, etc.

[00120] In one embodiment, the nanoparticle can be a core/shell nanoparticle which typically comprises a core nanoparticle surrounded by at least one shell. For example, the core/shell nanoparticle can be surrounded by an inner and outer shell. In another embodiment, the nanoparticle is a core nanoparticle which has a core but no surrounding shell. The outmost surface of the core or shell can be coated with tightly associated ligands which are not removed by ordinary solvation.

[00121] Examples of a nanoparticle include a nanocrystal, such as a core/shell nanocrystal, plus any associated organic ligands (which are not removed by ordinary solvation) or other materials which may coat the surface of the nanocrystal. In one embodiment, a nanoparticle has at least one major dimension ranging from about 1 to about 1000 nm. In other embodiments, a nanoparticle has at least one major dimension ranging from about 1 to about 20 nm, about 1 to about 15 nm, about 1 to about 10 nm or about 1 to 5 nm.

[00122] In some embodiments, a nanoparticle can have a layer of ligands on its surface which can further be cross-linked to each other. In some embodiments, a

nanoparticle can have other or additional surface coatings which can modify the properties of the particle, for example, increasing or decreasing solubility in water or other solvents. Such layers on the surface are included in the term ‘nanoparticle.’

[00123] In one embodiment, nanoparticle can refer to a nanocrystal having a crystalline core, or to a core/shell nanocrystal, and may be about 1 nm to about 100 nm in its largest dimension, about 1 nm to about 20 nm, about 1 nm to about 15 nm, about 1 nm to about 10nm or preferably about 5 nm to about 10nm in its largest dimension. Small nanoparticles are typically less than about 20nm in their largest dimension.

[00124] “Nanocrystal” as used herein can refer to a nanoparticle made out of an inorganic substance that typically has an ordered crystalline structure. It can refer to a nanocrystal having a crystalline core (core nanocrystal) or to a core/shell nanocrystal.

[00125] A core nanocrystal is a nanocrystal to which no shell has been applied. Typically, it is a semiconductor nanocrystal that includes a single semiconductor material. It can have a homogeneous composition or its composition can vary with depth inside the nanocrystal.

[00126] A core/shell nanocrystal is a nanocrystal that includes a core nanocrystal and a shell disposed over the core nanocrystal. Typically, the shell is a semiconductor shell that includes a single semiconductor material. In some embodiments, the core and the shell of a core/shell nanocrystal are composed of different semiconductor materials, meaning that at least one atom type of a binary semiconductor material of the core of a core/shell is different from the atom types in the shell of the core/shell nanocrystal.

[00127] The semiconductor nanocrystal core can be composed of a semiconductor material (including binary, ternary and quaternary mixtures thereof), from: Groups II-VI of the periodic table, including ZnS, ZnSe, ZnTe, CdS, CdSe, CdTe, HgS, HgSe, HgTe, MgTe; Groups III-V, including GaN, GaP, GaAs, GaSb, InN, InP, InAs, InSb, AlAs, AlP, AlSb, AlS; and/or Group IV, including Ge, Si, Pb.

[00128] The semiconductor nanocrystal shell can be composed of materials (including binary, ternary and quaternary mixtures thereof) comprising: ZnO, ZnS,

ZnSe, ZnTe, CdO, CdS, CdSe, CdTe, MgS, MgSe, GaAs, GaN, GaP, GaAs, GaSb, HgO, HgS, HgSe, HgTe, InAs, InN, InP, InSb, AlAs, AlN, AlP, or AlSb.

[00129] Many types of nanocrystals are known, and any suitable method for making a nanocrystal core and applying a shell to the core may be employed. Nanocrystals can have a surface layer of ligands to protect the nanocrystal from degradation in use or during storage.

[00130] “Quantum dot” as used herein refers to a crystalline nanoparticle made from a material which in the bulk is a semiconductor or insulating material, which has a tunable photophysical property in the near ultraviolet (UV) to far infrared (IR) range.

[00131] As used herein, the term “interaction” and its variants comprise any selective or specific interaction between a biomolecule and one or more targets, including but not limited to approach of the biomolecule to the target, transmission of an electrical, optical, chemical or other impulse between a biomolecule and a target, and/or binding of the biomolecule with the target. Optionally, the interaction can involve the formation of one or more bonds between the biomolecule and a target including, without limitation covalent, ionic, hydrogen, hydrophilic, hydrophobic, or affinity bonding as well as bonding or associations involving van der Waals forces and mechanical bonding. Some exemplary biomolecule-target interactions can include, for example, approach of the biomolecule and target to each other, movement of the biomolecule and target away from each other, association or dissociation of the biomolecule and target with each other, formation of a linkage between the biomolecule and target, transmission of one or more signals between the biomolecule and the target, independent binding of the biomolecule and target to a common entity or surface, activation of either the biomolecule or target by the other; etc.

[00132] Disclosed herein is a labeled biomolecule conjugate comprising: a biomolecule linked to a label to form a labeled biomolecule conjugate, wherein the conjugate has biological activity. Typically, the biological activity is an activity that is characteristic of the biomolecule.

[00133] In some embodiments, the label of the labeled biomolecule conjugate emits, or is capable of emitting, a signal. In some embodiments, the label of the labeled biomolecule conjugate induces, or is capable of inducing, the emission of a signal by another label. In some embodiments, the label of the conjugate is positioned to emit a

signal during interaction of the biomolecule with a target. Optionally, the signal indicates occurrence of the interaction. In some embodiments, the signal can indicate the identity of the target. Optionally, the signal can be detected to visualize and/or track the conjugate in real time.

[00134] In some embodiments, the biomolecule of the conjugate is capable of undergoing one or more transient interactions with a target, and the label of the conjugate is capable of emitting, or causing to be emitted, a signal during each of the one or more transient interactions. The one or more interactions can occur successively or simultaneously, and can involve one or multiple targets.

[00135] In some embodiments, the label of the conjugate is capable of emitting or inducing the emission of a series of signals, each signal corresponding to a transient interaction between the biomolecule and a target. The transient interactions can occur successively or simultaneously, and can involve one or multiple targets.

[00136] In some embodiments, the biomolecule can be selected from the group consisting of: a protein, a carbohydrate, a lipid, a nucleotide and a nucleic acid. In a typical embodiment, the biomolecule is an enzyme, even more typically a polymerase.

[00137] In some embodiments, the label of the conjugate can be selected from the group consisting of a nanoparticle and an organic dye. In some embodiments, the label is a fluorescent label. Optionally, the label is a fluorescent dye. The dye can be selected from the group consisting of: Cy3, ALEXA FLUOR, and fluorescein. In some embodiments, the nanoparticle can be a nanocrystal, typically a quantum dot.

[00138] In some embodiments, the biomolecule comprises an enzyme or a biologically active fragment thereof, the target is an enzyme substrate, the one or more transient interactions include one or more enzyme-mediated reactions. Such conjugates are referred to herein as labeled enzyme conjugates.

[00139] In some embodiments, the disclosure relates to a labeled enzyme conjugate comprising: an enzyme linked to at least one label to form a labeled enzyme conjugate. Optionally, the conjugate has enzymatic activity. Optionally, the enzyme is linked to two, three, four, five, six, seven or more detectable labels. In some embodiments, the enzymatic activity of the conjugate is at least about 1% relative to the enzymatic activity of the unconjugated enzyme. Optionally, the enzymatic

activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative to the polymerase activity of the unconjugated enzyme.

[00140] In some embodiments, the enzyme can include one or more attachment sites for the plurality of labels. The enzyme can be engineered or otherwise modified to include the one or more attachment sites. Optionally, the labels can be same; alternatively, at least two of the labels can be different from each other. In some embodiments, the plurality of labels are linked to a single attachment site on the enzyme. Alternatively, two or more of the plurality of labels can be linked to different attachment sites on the enzyme.

[00141] In some embodiments, the enzyme further comprises a modification enzyme recognition sequence. Optionally, the modification enzyme recognition sequence comprises a biotin ligase modification site.

[00142] In some embodiments, the disclosure relates to a labeled enzyme conjugate, comprising: a enzyme linked to one or more labels, where the conjugate has enzymatic activity and emits upon continuous excitation a total photon count of at least 10^2 photons before irreversibly photobleaching. In some embodiments, the conjugate emits a total photon count of at least 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 or 10^9 photons as measured using a test detection system. Optionally, the enzyme is linked to two, three, four, five, six, seven or more detectable labels. In some embodiments, the enzymatic activity of the conjugate is at least about 1% relative to the enzymatic activity of the unconjugated enzyme. Optionally, the enzymatic activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative to the polymerase activity of the unconjugated enzyme.

[00143] In some embodiments, the disclosure relates to a labeled enzyme conjugate, comprising: a first member of a binding pair linked to a enzyme; and a second member of the binding pair linked to at least one label, where the first and the second member of the binding pair are linked to each other to form a labeled enzyme conjugate having enzymatic activity. Optionally, the enzyme is linked to two, three,

four, five, six, seven or more detectable labels. In some embodiments, the enzymatic activity of the conjugate is at least about 1% relative to the enzymatic activity of the unconjugated enzyme. Optionally, the enzymatic activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative to the polymerase activity of the unconjugated enzyme. Optionally, the first member comprises a biotin moiety and the second member comprises an avidin moiety. In some embodiments, the second member is linked to at least one label. In some embodiments, at least one label generates a signal. In some embodiments, the at least one label is positioned to undergo FRET with a labeled nucleotide bound to an active site of the enzyme. In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the nucleotide label can be linked to the terminal phosphate of the polyphosphate. In some embodiments, the energy transfer produces a signal that can be detected in a single molecule reaction using a test detection system.

[00144] In some embodiments, the disclosure relates to a method for creating a labeled enzyme conjugate, comprising linking a enzyme to a plurality of labels to form a labeled enzyme conjugate having enzymatic activity.

[00145] Optionally, the linking further comprises linking the plurality of labels to an attachment site on the enzyme. Optionally, the plurality of labels are linked to independent attachment sites on the enzyme.

[00146] In some embodiments, the linking further comprises linking the enzyme to at least three labels.

[00147] In some embodiments, the linking further comprises linking a enzyme including a modification enzyme recognition sequence to the plurality of labels. Optionally, the modification enzyme recognition sequence comprises a biotin ligase modification site. Optionally, the linking further comprises linking a biotin moiety to the enzyme to produce a biotinylated enzyme.

[00148] Optionally, the method further comprises contacting the biotinylated enzyme with an avidin moiety linked to the plurality of labels under conditions where the avidin moiety binds to the biotin moiety, thereby forming a labeled enzyme conjugate including the enzyme linked to the plurality of labels and having enzymatic activity.

[00149] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of activity of the enzyme on a substrate. In some embodiments, the enzyme of the labeled enzyme conjugate is a nucleotide polymerase, the substrate is a labeled nucleotide, and the polymerase is linked to a label to form a labeled polymerase conjugate.

[00150] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of incorporation of the labeled nucleotide by the polymerase of the conjugate.

[00151] In some embodiments, the label of the labeled enzyme conjugate is a RET moiety positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme. In some embodiments, the enzyme of the labeled enzyme conjugate is a nucleotide polymerase and the substrate is a labeled nucleotide.

[00152] In some embodiments, the label of the labeled enzyme conjugate is a RET moiety positioned to undergo RET with a labeled substrate bound to the active site of the enzyme.

[00153] In some embodiments, the enzyme of the labeled enzyme conjugate is capable of undergoing one or more transient interactions with a substrate. In some embodiments, the enzyme is capable of undergoing multiple transient interactions with one or more substrates, which can occur simultaneously or successively.

[00154] In some embodiments, the enzyme is capable of undergoing transient interactions with one or more substrates, which can occur simultaneously or successively.

[00155] In some embodiments, the enzyme of the labeled enzyme conjugate is capable of undergoing transient interactions with a plurality of substrates, and the label of the conjugate is capable of generating a signal upon each interaction.

[00156] Optionally, the signal can be detected and analyzed to determine the identity of the substrate. In some embodiments, the enzyme of the conjugate is capable of undergoing transient interaction with a series of substrates in succession and the label of the conjugate is capable of producing a series of signals that can be detected and analyzed to determine a time series of interactions.

[00157] In some embodiments, a label of the labeled enzyme conjugate is a fluorescent label. In some embodiments, the fluorescent label can comprise a dye

selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein, and the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase.

[00158] In some embodiments, the polymerase of the labeled polymerase conjugate is an isolated variant of a naturally occurring polymerase, wherein the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 98% or 99% identical to an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36.

[00159] In some embodiments, the enzyme of the labeled enzyme conjugate is linked to the label through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond. In some embodiments, the bond is a covalent bond formed between an amine group of a lysine residue of the enzyme and an amine-reactive moiety, wherein the amine reactive moiety is linked to the label. In some embodiments, the bond is a covalent bond formed between a carboxy group of an amino acid residue of the enzyme and a maleimide moiety, wherein the maleimide moiety is linked to the label.

[00160] In some embodiments, an attachment moiety serves to link the enzyme to the label. In one exemplary embodiment, the labeled enzyme conjugate comprises an enzyme linked to one or more labels through an attachment moiety. Typically, the polymerase is linked to the attachment moiety, and the attachment moiety is linked to the one or more labels to form a labeled polymerase conjugate. In some embodiments, the attachment moiety of is an avidin moiety, and the enzyme comprises a biotin moiety, and the enzyme and the attachment moiety are linked to each other through a further biotin-avidin bond. In some embodiments, the attachment moiety is covalently attached to the one or more labels. In some embodiments, the one or more labels each comprises a biotin moiety, and the attachment moiety is linked to the one or more labels through a second biotin-avidin bond. In some embodiments, the attachment moiety is linked to two, three, four, five, six, seven, eight, nine, ten or more labels. In some embodiments, at least two of the labels are different from each other. In some embodiments, at least two of the labels

are the same. In some embodiments, at least two of the labels are positioned to undergo FRET with each other.

[00161] Also provided herein is a labeled enzyme conjugate, comprising: an enzyme linked to an attachment moiety, wherein the attachment moiety is linked to a label, thereby linking the enzyme to the label to form a labeled enzyme conjugate. Optionally, the attachment moiety can comprise a biotin moiety.

[00162] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of activity of the enzyme on a substrate. In some embodiments, the enzyme is a nucleotide polymerase and the substrate is a labeled nucleotide.

[00163] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of incorporation of the labeled nucleotide by the polymerase of the conjugate. In some embodiments, the label is a RET moiety positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme. In some embodiments, the enzyme is a nucleotide polymerase and the substrate is a labeled nucleotide.

[00164] In some embodiments, the label of the labeled enzyme conjugates or the labeled polymerase conjugates disclosed herein is a nanoparticle. In some embodiments, the label is a fluorescent label. In some embodiments, the fluorescent label comprises a dye selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein, and the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase.

[00165] In some embodiments, the polymerase is an isolated variant of a naturally occurring polymerase, wherein the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 98% or 99% identical to an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36.

[00166] In some embodiments, the enzyme is linked to the label through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond. In some embodiments, the bond is a covalent bond formed between an

amine group of a lysine residue of the enzyme and an amine-reactive moiety, wherein the amine reactive moiety is linked to the label. In some embodiments, the bond is a covalent bond formed between a carboxy group of an amino acid residue of the enzyme and a maleimide moiety, wherein the maleimide moiety is linked to the label.

[00167] In some embodiments, the attachment moiety of the labeled enzyme conjugate is an avidin moiety, the label and the enzyme are each linked to one or more biotin moieties, and the avidin moiety is linked to the enzyme and to the label through a biotin-avidin bond. In some embodiments, the attachment moiety is linked to two, three, four, five, six, seven, eight, nine, ten or more detectable labels. In some embodiments, at least two of the detectable labels are different from each other. In some embodiments, at least two of the detectable labels are the same. In some embodiments, at least two of the detectable labels are configured to undergo FRET with each other.

[00168] Also provided herein is a labeled enzyme conjugate, comprising: an enzyme linked to a first member of a binding pair; and a second member of the binding pair linked to a label; wherein the first member and the second member of the binding pair are linked to each other, thereby forming a labeled enzyme conjugate.

[00169] Also provided herein is a labeled enzyme conjugate, comprising: a first member of a binding pair linked to an enzyme; and a second member of the binding pair linked to a label; wherein the first member and the second member of the binding pair are linked to each other to form a labeled enzyme conjugate.

[00170] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of activity of the enzyme on a substrate. In some embodiments, the enzyme of the labeled enzyme conjugate is a nucleotide polymerase and the substrate is a labeled nucleotide. In some embodiments, the label is positioned to emit a detectable signal indicative of incorporation of the labeled nucleotide by the polymerase of the conjugate.

[00171] In some embodiments, the label of the labeled enzyme conjugate is a RET moiety positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme. In some embodiments, the enzyme is a nucleotide polymerase and the substrate is a labeled nucleotide. In some embodiments, the second member of the binding pair is linked to two or more detectable labels. In

some embodiments, at least two of the two or more detectable labels are different from each other.

[00172] In some embodiments, the binding pair is selected from the group consisting of: an avidin moiety and a biotin moiety; an antigenic epitope and an antibody or immunologically reactive fragment thereof; an antibody and a hapten; a digoxigen moiety and an anti-digoxigen antibody; a fluorescein moiety and an anti-fluorescein antibody; an operator and a repressor; a nuclease and a nucleotide; a lectin and a polysaccharide; a steroid and a steroid-binding protein; an active compound and an active compound receptor; a hormone and a hormone receptor; an enzyme and a substrate; an immunoglobulin and protein A; and an oligonucleotide or polynucleotide and its corresponding complement.

[00173] In some embodiments, the label is a fluorescent label. In some embodiments, the fluorescent label comprises a dye selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein, and the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase.

[00174] In some embodiments, the label is a nanoparticle. In some embodiments, the polymerase is an isolated variant of a naturally occurring polymerase, wherein the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 98% or 99% identical to an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36.

[00175] Also disclosed herein is a method of making a labeled enzyme conjugate, comprising: (a) linking a first member of a binding pair to an enzyme to form an enzyme binding conjugate; and (b) contacting the enzyme binding conjugate with a second member of the binding pair, wherein the second member is linked to one or more detectable labels, and wherein said contacting is performed under conditions where the first and second members of the binding pair become linked to each other, thereby forming a labeled enzyme conjugate comprising an enzyme linked to one or more labels.

[00176] In some embodiments of the method, the label is a RET moiety and is positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme.

[00177] In some embodiments of the method, the enzyme is a nucleotide polymerase and the substrate is a labeled nucleotide.

[00178] In some embodiments, the binding pair is selected from the group consisting of: an avidin moiety and a biotin moiety; an antigenic epitope and an antibody or immunologically reactive fragment thereof; an antibody and a hapten; a digoxigen moiety and an anti-digoxigen antibody; a fluorescein moiety and an anti-fluorescein antibody; an operator and a repressor; a nuclease and a nucleotide; a lectin and a polysaccharide; a steroid and a steroid-binding protein; an active compound and an active compound receptor; a hormone and a hormone receptor; an enzyme and a substrate; an immunoglobulin and protein A; and an oligonucleotide or polynucleotide and its corresponding complement.

[00179] In some embodiments, the enzyme of the labeled enzyme conjugate is linked to the one or more labels of the conjugate through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond.

[00180] In some embodiments, the first member of the binding pair is a biotin moiety and the second member of the binding pair comprises a streptavidin moiety.

[00181] Also disclosed herein is a method of making a labeled enzyme conjugate, comprising: (a) linking a first member of a binding pair to an enzyme; (b) linking the second member of the binding pair to one or more labels, and (c) contacting the products of steps (a) and (b) with each other under conditions where the first member and second members of the binding pair become linked to each other to form a labeled enzyme conjugate comprising an enzyme linked to the one or more labels, where the conjugate has enzymatic activity.

[00182] In some embodiments, the first member of the binding pair is a biotin moiety and the second member of the binding pair comprises a streptavidin moiety.

[00183] Also disclosed herein is a labeled enzyme conjugate for use in single molecule reactions prepared by the above methods. In some embodiments, the labeled enzyme conjugate comprises a first member of a binding pair linked to an enzyme; and

a second member of the binding pair linked to at least one label, where the first and the second member of the binding pair are linked to each other to form a labeled enzyme conjugate having enzymatic activity.

[00184] Optionally, the first member comprises a biotin moiety and the second member comprises an avidin moiety. Optionally, the second member of the binding pair is linked to two, three, four, five, six, seven or more detectable labels. In some embodiments, the enzymatic activity of the conjugate is at least about 1% relative to the enzymatic activity of the unconjugated enzyme. Optionally, the enzymatic activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative to the enzymatic activity of the unconjugated enzyme.

[00185] In some embodiments, at least one label generates a signal. In some embodiments, the at least one label is positioned to undergo FRET with a labeled substrate bound to an active site of the enzyme. In some embodiments, the labeled nucleotide comprises a polyphosphate. In some embodiments, the energy transfer produces a signal that can be detected in a single molecule reaction using a test detection system.

[00186] Also provided herein is a labeled enzyme conjugate, comprising: an enzyme linked to at least two detectable labels to form a labeled enzyme conjugate, wherein the enzyme of the conjugate has enzymatic activity.

[00187] In some embodiments, the label of the labeled enzyme conjugate is positioned to emit a detectable signal indicative of activity of the enzyme on a substrate.

[00188] In some embodiments, the enzyme is a nucleotide polymerase and the substrate is a labeled nucleotide.

[00189] In some embodiments, the label is positioned to emit a detectable signal indicative of incorporation of the labeled nucleotide by the polymerase of the conjugate.

[00190] In some embodiments, the label is a RET moiety positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme.

[00191] In some embodiments, the label comprises a fluorescent label. In some embodiments, the fluorescent label comprises a dye selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein, and the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase. In some embodiments, the label comprises a nanoparticle.

[00192] In some embodiments, the polymerase is an isolated variant of a naturally occurring polymerase, wherein the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 98% or 99% identical to an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36. In some embodiments, the enzyme is linked to the label through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond. In some embodiments, the bond is a covalent bond formed between an amine group of a lysine residue of the enzyme and an amine-reactive moiety, wherein the amine reactive moiety is linked to the label. In some embodiments, the bond is a covalent bond formed between a carboxy group of an amino acid residue of the enzyme and a maleimide moiety, wherein the maleimide moiety is linked to the label.

[00193] In some embodiments, the enzyme of the labeled enzyme conjugate is linked to an attachment moiety, and wherein the attachment moiety is linked to the at least two detectable labels. In some embodiments, at least two of the detectable labels are different from each other. In some embodiments, at least two of the detectable labels are configured to undergo FRET with each other.

[00194] Also disclosed herein is a labeled enzyme conjugate for use in single molecule polymerization reactions.

[00195] Also disclosed herein is a system for monitoring successive enzyme-substrate interactions, comprising: one or more substrates; an enzyme that undergoes one or more transient interactions with the one or more substrates, and a label linked to the enzyme, wherein the label emits, or causes to be emitted, one or more detectable signals upon a transient interaction of the enzyme with the one or more substrates.

[00196] Also disclosed herein is a system for monitoring successive interactions of an enzyme (e.g., a polymerase), with one or more targets (e.g., nucleotides), comprising: a target; a labeled enzyme conjugate comprising an enzyme linked to at least one label, where the conjugate undergoes, or is capable of undergoing, a transient interaction with the target, and where the label of the conjugate is capable of emitting or inducing the emission of a signal upon each such transient interaction. In some embodiments, the label of the conjugate emits or induces the emission of a signal upon each such transient interaction. Optionally, the signal can be detected and analyzed to determine the identity of the target.

[00197] Optionally, the the conjugate can undergo multiple transient interactions with the target, which can occur simultaneously or successively. In some embodiments, the conjugate undergoes a series of transient interaction with a series of targets in succession, and the label is capable of emitting (or inducing the emission of) a series of signals that can be detected and analyzed to determine a time series of interactions.

[00198] In some embodiments, the target is a labeled nucleotide. In some embodiments, the nucleotide label is bonded to a portion of the nucleotide that is released during incorporation of the nucleotide. Optionally, the nucleotide comprises a polyphosphate chain that is released during incorporation, and the nucleotide label is bonded to the beta, gamma or other terminal phosphate of the labeled nucleotide.

[00199] In some embodiments, the biomolecule of the system comprises a polymerase, and the at least two transient and successive interactions comprise nucleotide incorporations.

[00200] In some embodiments, the enzyme of the labeled enzyme conjugate is a polymerase, the target is a nucleotide, and the one or more transient interactions each comprises a nucleotide incorporation catalyzed by the polymerase. When the biomolecule of the conjugate is a polymerase, the conjugate is typically referred as a "labeled polymerase conjugate".

[00201] One exemplary embodiment of the present disclosure is a labeled polymerase conjugate comprising a polymerase linked to a label. The conjugate can have polymerase activity. In some embodiments, the label can be a nanoparticle. In some embodiments, the label can be an organic dye.

[00202] In some embodiments, the disclosure relates to a labeled polymerase conjugate comprising a polymerase linked to a plurality of labels. The conjugate can have polymerase activity.

[00203] Optionally, the polymerase activity of the conjugate is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97% or 99% relative to the polymerase activity of the unconjugated polymerase.

[00204] In some embodiments, the polymerase can be linked to at least three, four, five, six, seven or more labels.

[00205] In some embodiments, the label of the labeled polymerase conjugate is positioned to emit a signal during the interaction of the polymerase with a nucleotide. Optionally, the interaction comprises the incorporation of the nucleotide into a nucleic acid molecule by the polymerase. Optionally, the signal indicates the occurrence of the nucleotide incorporation. In some embodiments, the signal can indicate the identity of the nucleotide that is incorporated. Optionally, the signal can be detected to visualize and/or track the conjugate in real time. In some embodiments, a signal indicative of nucleotide incorporation is generated as each incoming nucleotide becomes incorporated by the polymerase of the conjugate.

[00206] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising a polymerase linked to a plurality of labels to form a labeled polymerase conjugate, where the conjugate has polymerase activity, and where at least one label of the conjugate performs energy transfer with a labeled nucleotide bound to an active site of the polymerase.

[00207] In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the nucleotide label can be linked to the terminal phosphate of the polyphosphate.

[00208] In some embodiments, the energy transfer produces a signal that can be detected in a single molecule reaction using a test detection system.

[00209] In some embodiments, the polymerase can include one or more attachment sites for the plurality of labels. The polymerase can be engineered or otherwise modified to include the one or more attachment sites. Optionally, the labels can be same; alternatively, at least two of the labels can be different from each other. In some embodiments, the plurality of labels are linked to a single attachment site on the

polymerase. Alternatively, two or more of the plurality of labels can be linked to different attachment sites on the polymerase. Optionally, the plurality of labels includes three, four, five, six, seven or more labels. Optionally, the polymerase comprises a modification enzyme recognition sequence.

[00210] In some embodiments, the label of the labeled polymerase conjugate is positioned to emit a detectable signal indicative of incorporation of a labeled nucleotide by the polymerase of the conjugate.

[00211] In some embodiments, the label of the conjugate is a RET moiety positioned to undergo RET with the label of a labeled nucleotide bound to an active site of the polymerase.

[00212] In some embodiments, the polymerase undergoes, or is capable of undergoing, one or more transient interactions with a nucleotide. Optionally, such transient interactions can occur simultaneously or successively, and can involve the same or different nucleotides.

[00213] In some embodiments, the polymerase undergoes, or is capable of undergoing, transient interactions with a plurality of nucleotides in succession, and the label of the conjugate generates, or is capable of generating, a signal upon each such interaction. Optionally, the signal can be detected and analyzed to determine the identity of the incorporated nucleotide. In some embodiments, the polymerase undergoes, or is capable of undergoing, transient interactions with a series of substrates in succession and the label emits or induces, or is capable of emitting or inducing, a series of detectable signals that can be detected and analyzed to determine a time series of interactions.

[00214] In some embodiments, the label of the labeled polymerase conjugate is a fluorescent label. In some embodiments, the fluorescent label can comprise a dye selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein. Optionally, the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase.

[00215] In some embodiments, the conjugate may optionally comprise a polymerase linked to a label through a linker or chemical linkage comprising a bond selected from the group consisting of: a covalent bond, an electrostatic bond and an affinity

bond. In some embodiments, the linker or chemical linkage comprises a bond through a functional group, including, without limitation, a hydroxyl, a carboxyl, a carbonyl, a sulfhydryl, an amine, an amide, a nitrile, a nitrogen with a free lone pair of electrons, an amino acid, a thiol, a sulfonic acid, a sulfonyl halide, and an acyl halide.

[00216] In some embodiments, the labeled polymerase conjugate comprises a polymerase linked to the label through a covalent bond. The covalent bond can be formed using any suitable method, optionally including through use of crosslinking agents or linkers.

[00217] Optionally, the nucleotide comprises a label (referred to herein as “a nucleotide label”). The label can optionally be bonded to a portion of the nucleotide that is released during nucleotide incorporation. By releasing the label upon incorporation, successive extensions can each be detected without interference from nucleotides previously incorporated into the complementary strand.

[00218] In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the nucleotide label can be linked to the terminal phosphate of the polyphosphate. In some embodiments, the terminal phosphate is a beta or gamma phosphate.

[00219] In some embodiments, the polymerase of the labeled polymerase conjugate comprises one member of a binding pair and the label comprises a complementary member of the binding pair.

[00220] Optionally, the polymerase of the conjugate is a DNA polymerase. In some embodiments, the DNA polymerase is at least 95% identical to a DNA polymerase selected from the group consisting of: Phi-29 DNA polymerase, B103 DNA polymerase, the Klenow fragment of *E. coli* DNA polymerase and HIV reverse transcriptase.

[00221] Optionally, the label of the conjugate is positioned relative to the polymerase to perform an energy transfer reaction. In some embodiments, the label is positioned to perform FRET with a labeled nucleotide bound to the nucleotide binding site of the polymerase. Optionally, the label of the conjugate is positioned to perform FRET with a label linked to the terminal phosphate of a polyphosphate-comprising nucleotide bound to an active site of the polymerase. Optionally, the label of the

conjugate undergoes FRET with the nucleotide label with a FRET efficiency of at least about 20%.

[00222] In other embodiments, the polymerase is a mutant or variant Phi-29 DNA polymerase comprising an N-terminal polyhistidine tag (His-tag) fused to an amino acid sequence at least 85% identical to a Phi-29 DNA polymerase comprising the amino acid sequence of SEQ ID NO: 3, or any biologically active fragment thereof.

[00223] In some embodiments, the disclosure relates to a method for creating a labeled polymerase conjugate, comprising linking a polymerase to a plurality of labels to form a labeled polymerase conjugate having polymerase activity.

[00224] Optionally, the linking further comprises linking the plurality of labels to an attachment site on the polymerase. Optionally, the plurality of labels are linked to independent attachment sites on the polymerase.

[00225] In some embodiments, the linking further comprises linking the polymerase to at least three labels.

[00226] In some embodiments, the linking further comprises linking a polymerase including a modification enzyme recognition sequence to the plurality of labels. Optionally, the modification enzyme recognition sequence comprises a biotin ligase modification site. Optionally, the linking further comprises linking a biotin moiety to the polymerase to produce a biotinylated polymerase.

[00227] Optionally, the method further comprises contacting the biotinylated polymerase with an avidin moiety linked to the plurality of labels under conditions where the avidin moiety binds to the biotin moiety, thereby forming a labeled polymerase conjugate including the polymerase linked to the plurality of labels and having polymerase activity.

[00228] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising: a first member of a binding pair linked to a polymerase; and a second member of the binding pair linked to at least one label, where the first and the second member of the binding pair are linked to each other to form a labeled polymerase conjugate having polymerase activity.

[00229] Optionally, the first member comprises a biotin moiety and the second member comprises an avidin moiety. In some embodiments, the second member is linked to at least one label. Optionally, the second member of the binding pair is

linked to two, three, four, five, six, seven or more detectable labels. In some embodiments, the enzymatic activity of the polymerase is at least about 1% relative to the polymerase activity of the unconjugated polymerase. Optionally, the polymerase activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative to the polymerase activity of the unconjugated polymerase.

[00230] In some embodiments, at least one label generates a signal. In some embodiments, the at least one label is positioned to undergo FRET with a labeled nucleotide bound to an active site of the polymerase. In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the nucleotide label can be linked to the terminal phosphate of the polyphosphate. In some embodiments, the energy transfer produces a signal that can be detected in a single molecule reaction using a test detection system.

[00231] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising: a polymerase linked to one or more labels, where the conjugate has polymerase activity and emits upon continuous excitation a total photon count of at least 10^2 photons before irreversibly photobleaching. In some embodiments, the conjugate emits a total photon count of at least 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 or 10^9 photons as measured using a test detection system. Optionally, the polymerase is linked to at least three labels.

[00232] In some embodiments, the disclosure relates to a labeled polymerase conjugate, comprising: a first member of a binding pair linked to a polymerase; and a second member of the binding pair linked to at least one label, where the first and the second member of the binding pair are linked to each other to form a labeled polymerase conjugate having polymerase activity.

[00233] Optionally, the first member comprises a biotin moiety and the second member comprises an avidin moiety. In some embodiments, the second member is linked to at least one label. In some embodiments, at least one label generates a signal. In some embodiments, the at least one label is positioned to undergo FRET with a labeled nucleotide bound to an active site of the polymerase. In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the

nucleotide label can be linked to the terminal phosphate of the polyphosphate. In some embodiments, the energy transfer produces a signal that can be detected in a single molecule reaction using a test detection system.

[00234] Also disclosed herein is a method for performing nucleotide incorporation, comprising: contacting a labeled polymerase conjugate including a polymerase linked to a label with a nucleotide under conditions where the polymerase catalyzes incorporation of the nucleotide into a nucleic acid molecule. Optionally, the nucleotide comprises a label (referred to herein as “a nucleotide label”). The label can optionally be bonded to a portion of the nucleotide that is released during nucleotide incorporation. By releasing the label upon incorporation, successive extensions can each be detected without interference from nucleotides previously incorporated into the complementary strand.

[00235] In some embodiments, the labeled nucleotide comprises a polyphosphate. Optionally, the nucleotide label can be linked to the terminal phosphate of the polyphosphate. In some embodiments, the terminal phosphate is a beta or gamma phosphate.

[00236] Also disclosed herein is a method for nucleotide incorporation, comprising: contacting the labeled polymerase conjugate as provided herein with one or more labeled nucleotides under conditions where a labeled nucleotide is incorporated into an extending nucleic acid molecule by the labeled polymerase.

[00237] Also disclosed herein is kit for use in single molecule sequencing reactions, comprising a labeled polymerase conjugate according to the present disclosure. In some embodiments, the kit further comprises labeled nucleotides.

[00238] Also disclosed herein is kit for use in single molecule sequencing reactions, comprising a labeled polymerase conjugate including a polymerase linked to at least one label, wherein the conjugate has polymerase activity. Optionally, the polymerase can be linked to at least three labels. In some embodiments, the kit further comprises labeled nucleotides.

[00239] Also disclosed herein is system for single molecule sequencing, comprising: (a) a reaction chamber wherein the one or more template nucleic acid molecules are contacted with a labeled polymerase conjugate and one or more labeled nucleotides under conditions where the one or more nucleotides are polymerized by the

polymerase onto the end of an extending nucleic acid molecule such that one or more detectable signals indicative of nucleotide incorporation are generated; (b) detection means for detecting the one or more detectable signals indicative of nucleotide incorporation; and (c) an analyzer for analyzing the one or more detected signals and converting them into nucleic acid sequence information. In some embodiments, the labeled polymerase conjugate comprises a polymerase linked to a label to form a labeled polymerase conjugate, wherein conjugate has polymerase activity. In some embodiments, the label of the labeled polymerase is positioned to emit a signal indicative of incorporation of the labeled nucleotide by the labeled polymerase. In some embodiments, the label of the labeled polymerase is positioned to emit a signal indicative of incorporation of the labeled nucleotide by the polymerase of the conjugate. In some embodiments, the detectable label of the labeled polymerase is a RET moiety positioned to undergo RET with the label of a labeled substrate positioned in the active site of the enzyme.

[00240] In some embodiments, the detectable label comprises a fluorescent label. In some embodiments, the fluorescent label comprises a dye selected from the group consisting of: Cy3, Cy3b, Alexa Fluors and fluorescein, and the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a variant of Phi-29 DNA polymerase, B103 DNA polymerase and a variant of B103 DNA polymerase. In some embodiments, the detectable label comprises a nanoparticle.

[00241] In some embodiments, the polymerase is an isolated variant of a naturally occurring polymerase, wherein the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 98% or 99% identical to an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36. In some embodiments, the enzyme is linked to the detectable label through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond. In some embodiments, the bond is a covalent bond formed between an amine group of a lysine residue of the enzyme and an amine-reactive moiety, wherein the amine reactive moiety is linked to the detectable label. In some embodiments, the bond is a covalent

bond formed between a carboxy group of an amino acid residue of the enzyme and a maleimide moiety, wherein the maleimide moiety is linked to the detectable label.

[00242] Also disclosed herein is system for single molecule sequencing, comprising: (a) a reaction chamber wherein a labeled polymerase conjugate including a polymerase linked to at least one label is contacted with at least one labeled nucleotide under conditions where the polymerase catalyzes the incorporation of the at least one labeled nucleotide such that a signal corresponding to each incorporation of a labeled nucleotide is generated; (b) a detector for detecting a time series of signals, each signal corresponding to each nucleotide incorporation; and (c) an analyzer to analyze the times series of signal to determine a sequence of nucleotide incorporations.

[00243] Provided herein are conjugate compositions comprising one or more biomolecules or biologically active fragments thereof operably linked to one or more nanoparticles, hereinafter referred to as “biomolecule/nanoparticle conjugates.” Compositions comprising labeled biomolecule conjugates of the present disclosure can be useful in a wide variety of biological applications. For example, such conjugates can allow direct visualization of the biomolecule of the conjugate. Optionally, the biomolecule can be visualized and/or tracked in real time. In some embodiments, such visualization can be done in real time or near real time, optionally in high throughput and/or single molecule format. Such visualization can permit, for example, detection and evaluation of a wide range of biomolecular behavior over an extended period both in *vivo* and *in vitro* contexts, including but not limited to biomolecular movement and/or transport within a cell or living organism, association or dissociation of different biomolecules, protein expression patterns within living cells or organisms, approach and/or binding of a biomolecule to a particular target, detection of movement as a function of biomolecular activity such as, for example, polymerase movement along a template, etc. See, e.g., Jaiswal et al., “Use of quantum dots for live cell imaging”, *Nature Methods*, 1(1):71-78. Observation of multiple different biomolecules or behaviors simultaneously can be achieved through use of different nanoparticles having different characteristic wavelengths, e.g., colors, and/or intensities.

[00244] Such conjugates can also be useful in applications requiring detection of biomolecular activity, including in single molecule and/or high-throughput format. For example, such conjugates can be useful in diagnostic assays involving detection of a signal generated as a result of biomolecular activity. In some embodiments, the biomolecule can be linked to the label such that the label is capable of functioning as a reporter of biomolecular activity in real time or near real time. Biomolecular activity can frequently involve interaction of the biomolecule with a specific target, such as, for example, the interaction of an enzyme with a substrate. Occasionally, the biomolecule is capable of undergoing interactions with multiple targets either successively or simultaneously. Elucidating the nature of such biomolecule-target interactions can be important in determining the biological function of the biomolecule. Studies of such interactions have traditionally involved use of a labeled target, which is frequently degraded as a result of the interaction. This problem can be avoided by conjugating a label, e.g., a nanoparticle or an organic dye moiety, directly to the biomolecule. Such conjugation can allow for direct visualization of individual biomolecules, as well as the monitoring of multiple interactions of a biomolecule with multiple targets over time.

[00245] In some embodiments, the conjugates can permit not only visualization but also manipulation and sorting of biomolecules within a large population. For example, in some embodiments the conjugates can be sorted using suitable optical manipulation techniques such as “optical tweezers”. See, e.g., Jauffred et al., “Three-dimensional optical control of individual quantum dots”, *Nano Lett.* 8(10):3376-3380 (2008).

[00246] The labeled polymerase conjugates disclosed herein can be advantageously employed in the sequencing methods described in U.S. Patent No. 7,329,492 to Hardin et al.; U.S. Patent No. 6, 982, 146 to Schneider et al. The superior photostability and/or signal strength of the polymerase conjugates provided herein can be used to produce superior read length or accuracy in such single molecule sequence methods employing FRET between a labeled polymerase and labeled nucleotide.

[00247] Some additional disclosures relating to methods of making labeled polymerase conjugates and to modified polymerases that can be used to make the

conjugates provided herein, are disclosed, for example, in U.S. provisional application nos. 61/184,770, filed June 5, 2009; 61/245,457, filed on September 24, 2009; 61/299,919, filed on January 29, 2010; 61/242,771, filed on September 15, 2009; and 61/293,618, filed on January 8, 2010, as well as in U.S. Application No. ____ titled “Conjugates of Biomolecules to Nanoparticles” and assigned Attorney Docket No. LT00003, filed concurrently herewith; and U.S. Application No. ____ titled “Polymerase Compositions & Methods” and assigned Attorney Docket No. LT00052, filed concurrently herewith.

[00248] In some embodiments, the label of the labeled biomolecule conjugate comprises a nanoparticle, and the labeled biomolecule conjugate comprises a biomolecule or biologically active fragment thereof linked to a nanoparticle (a type of conjugate referred to herein as a “biomolecule/nanoparticle conjugate”). The superior detectability of nanoparticles as compared to conventional organic dye molecules can allow for increased signal in high-throughput in single molecule applications. Additionally, the conjugates of the present disclosure can be useful in highly multiplexed applications. For example, the size-tunable emission properties of labeled biomolecule conjugates comprising nanoparticle-based labels can also be exploited to design assays involving wavelength and/or intensity multiplexing. Such conjugates can be useful in, e.g., performing multiple optical coding for biological assays. For example, the use of 10 different intensity levels and 6 colors could theoretically be used to code one million different biomolecules, opening new opportunities in gene expression, high throughput, diagnostic and other biological applications. See, e.g., Han et al., “Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules”, *Nat. Biotech.* 19:631-635 (2001).

[00249] In some embodiments, the surfaces, labels (including, e.g., nanoparticles and organic dyes), polymerases, nucleotides and nucleic acid molecules (including, e.g., targets, primers and/or oligonucleotides) of the present disclosure can be linked to each other, in any combination and in any order, using well known linking chemistries. Such linkage can optionally include a covalent bond and/or a non-covalent bond selected from the group consisting of an ionic bond, a hydrogen bond, an affinity bond, a dipole-dipole bond, a van der Waals bond, and a hydrophobic bond.

In some embodiments, the linking procedure used to link the biomolecules, labels and/or surfaces of the present disclosure comprises a chemical reaction that includes formation of one or more covalent bonds between a first and second moiety, resulting in the linkage of the first moiety to the second moiety. In some embodiments, the chemical reaction occurs between a first group of the moiety and a second group of the second moiety. Such chemical reaction can include, for example, reaction of activated esters, acyl azides, acyl halides, acyl nitriles, or carboxylic acids with amines or anilines to form carboxamide bonds. Reaction of acrylamides, alkyl halides, alkyl sulfonates, aziridines, haloacetamides, or maleimides with thiols to form thioether bonds. Reaction of acyl halides, acyl nitriles, anhydrides, or carboxylic acids with alcohols or phenols to form an ester bond. Reaction of an aldehyde with an amine or aniline to form an imine bond. Reaction of an aldehyde or ketone with a hydrazine to form a hydrazone bond. Reaction of an aldehyde or ketone with a hydroxylamine to form an oxime bond. Reaction of an alkyl halide with an amine or aniline to form an alkyl amine bond. Reaction of alkyl halides, alkyl sulfonates, diazoalkanes, or epoxides with carboxylic acids to form an ester bond. Reaction of an alkyl halides or alkyl sulfonates with an alcohol or phenol to form an ether bond. Reaction of an anhydride with an amine or aniline to form a carboxamide or imide bond. Reaction of an aryl halide with a thiol to form a thiophenol bond. Reaction of an aryl halide with an amine to form an aryl amine bond. Reaction of a boronate with a glycol to form a boronate ester bond. Reaction of a carboxylic acid with a hydrazine to form a hydrazide bond. Reaction of a carbodiimide with a carboxylic acid to form an N-acylurea or anhydride bond. Reaction of an epoxide with a thiol to form a thioether bond. Reaction of a haloplatinate with an amino or heterocyclic group to form a platinum complex. Reaction of a halotriazine with an amine or aniline to form an aminotriazine bond. Reaction of a halotriazines with an alcohol or phenol to form a triazinyl ether bond. Reaction of an imido ester with an amine or aniline to form an amidine bond. Reaction of an isocyanate with an amine or aniline to form a urea. Reaction of an isocyanate with an alcohol or phenol to form a urethane bond. Reaction of an isothiocyanate with an amine or aniline to form a thiourea bond. Reaction of a phosphoramidate with an alcohol to form a phosphite ester bond. Reaction of a silyl

halide with an alcohol to form a silyl ether bond. Reaction of a sulfonate ester with an amine or aniline to form an alkyl amine bond. Reaction of a sulfonyl halide with an amine or aniline to form a sulfonamide bond. Reaction of a thioester with thiol group of a cysteine followed by rearrangement to form an amide bond. Reaction of an azide with an alkyne to form a 1,2,3-triazole. Reaction of an aldehyde with an N-terminal cysteine to form a 5-membered thiazolidine ring.

[00250] In some embodiments, water-insoluble substances can be chemically modified in an aprotic solvent such as dimethylformamide, dimethylsulfoxide, acetone, ethyl acetate, toluene, or chloroform. Similar modification of water-soluble substances can be accomplished using reactive compounds to make them more readily soluble in organic solvents.

[00251] In some embodiments the biomolecules and/or labels of the present disclosure are linked to a surface. Optionally, such linkage can result in reversible or non-reversible immobilization of the nanoparticles, polymerases, nucleotides, nucleic acid molecules, primers, and/or oligonucleotides onto the surface. Non-limiting examples of such linkage can include: nucleic acid hybridization, protein aptamer-target binding, non-specific adsorption, and solvent evaporation. In some embodiments, the biomolecule that is linked to a surface is a polymerase (such as, for example, a polymerase fusion protein). The polymerase can be attached to a surface via a linker comprising an anchor or tethering moiety. The anchor or tethering moiety can be flexible or rigid. The anchor or tether can orient the polymerase, or polymerase fusion protein, in a manner that does not interfere with the nucleotide binding and/or polymerase activity.

[00252] Linkage of biomolecules to labels, surfaces and/or to each other can be accomplished by any suitable method (for example, Brinkley et al., 1992 *Bioconjugate Chem.* 3: 2). In some embodiments, a biomolecule can comprise a single type of reactive site (as is typical for polysaccharides), or it can comprise multiple types of reactive sites, e.g., amines, thiols, alcohols, phenols, may be available (as is typical for proteins). Conjugation selectivity can be obtained by selecting an appropriate reactive moiety. For example, modification of thiols with a thiol-selective reagent such as a haloacetamide or maleimide, or modification of amines with an amine-reactive reagent such as 1-ethyl-3-(3-dimethyl-aminopropyl)

carbodiimide (variously known as EDC or EDAC), an activated ester, acyl azide, isothiocyanate or 3,5-dichloro-2,4,6-triazine. Partial selectivity can also be obtained by careful control of the reaction conditions.

[00253] In some embodiments, the biomolecule of the labeled biomolecule conjugate is linked to the label through a bond selected from group consisting of: a covalent bond, a hydrogen bond, a hydrophilic bond, a hydrophobic bond, an electrostatic bond, a Van der Waals bond, and an affinity bond.

[00254] In some embodiments, the biomolecule comprises a peptide and the bond is a covalent bond formed between an amine group of a lysine residue of the biomolecule and an amine-reactive moiety, wherein the amine reactive moiety is linked to the label. In some embodiments, the biomolecule comprises a peptide and the bond is a covalent bond formed between a carboxy group of an amino acid residue of the biomolecule and a maleimide moiety, wherein the maleimide moiety is linked to the label.

[00255] In some embodiments, the label of the labeled biomolecule conjugate comprises a nanoparticle. Optionally, the nanoparticle further comprises a carboxyl group on its surface, and the one or more biomolecules or fragments a primary amine group, and the cross-linking agent EDC is employed to form a covalent amide bond between the nanoparticle and the one or more biomolecules or fragments.

[00256] In some embodiments, the biomolecule can be attached to label (including, e.g., a FRET donor or acceptor moiety) using any suitable chemical linking procedure, including chemical linking procedures that are known in the art. In some embodiments, the biomolecule or biologically active fragment can be linked to the nanoparticle via chemical linking procedures. Many linking procedures are well known in the art, including: maleimide, iodoacetyl, or pyridyl disulfide chemistry which targets thiol groups on polypeptides; or succinimidyl esters (NHS), sulfonyl chlorides, iso(thio)cyanates, or carbonyl azide chemistry which targets primary amines in a polypeptide, and dichlorotriazine-based linking procedures. Additional exemplary linking procedures are described in more detail herein.

[00257] In some embodiments, the appropriate reactive compounds can be dissolved in a nonhydroxylic solvent (usually DMSO or DMF) in an amount sufficient to give a suitable degree of conjugation when added to a solution of the protein to be

conjugated. These methods have been used to prepare protein conjugates from antibodies, antibody fragments, avidins, lectins, enzymes, proteins A and G, cellular proteins, albumins, histones, growth factors, hormones, and other proteins. The resulting protein (e.g., polymerase) attached to the energy transfer or reporter moiety can be used directly or enriched, e.g., chromatographically enriched to separate the desired linked compound from the undesired unlinked compound. Several linking procedures are described in U.S. Patents and 5,188,934. Other suitable linking procedures are also known in the art.

[00258] When conjugating biomolecules to nanoparticles, the residual, unreacted compound or a compound hydrolysis product can be removed by dialysis, chromatography or precipitation. The presence of residual, unconjugated moieties can be detected by methods such as thin layer chromatography which elutes the unconjugated forms away from its conjugate. In some embodiments, the reagents are kept concentrated to obtain adequate rates of conjugation.

[00259] In some embodiments, the surfaces, labels (including, e.g., dyes and/or nanoparticles) and/or biomolecules (including, e.g., polymerases, nucleotides and nucleic acid molecules) disclosed herein can be modified to facilitate their linkage to each other. Such modification can optionally include chemical or enzymatic modification. The modification can be practiced in any combination and in any order. In some embodiments, the modification can mediate covalent or non-covalent linkage of the surfaces, labels and/or biomolecules with each other.

[00260] In some embodiments, the biomolecule can be attached, fused or otherwise associated with a moiety that facilitates purification and/or isolation of the biomolecule. For example, the moiety can be a modification enzyme recognition site, an epitope or an affinity tag that facilitates purification of the biomolecule.

[00261] In some embodiments, the polymerase can include an amino acid analog which provides a reactive group for linking to the nanoparticle, target, substrate and/or surface. For example, the amino acid analog can be produced using a cell (e.g., bacterial cell) which is genetically engineered to have a 21 amino acid genetic code which is capable of inserting the amino acid analog into the encoded polymerase (or fusion protein). The inserted amino acid analog can be used in a linking

chemistry procedure to attach the polymerase (or fusion protein) to the energy transfer donor moiety, biomolecule or the surface.

[00262] In some embodiments, the biomolecule is a protein and is modified with a His tag. In some embodiments, the His tag may be fused directly with the protein; alternatively, a linker comprising various lengths of amino acid residues can be placed between the protein and the His tag. The linker can be flexible or rigid.

[00263] Optionally, the presence of the His tag can facilitate purification of the protein. For example, His tagged protein can be purified from a raw bacterial lysate by contacting the lysate with any suitable affinity medium comprising bound metal ions to which the histidine residues of the His-tag can bind, typically via chelation. The bound metal ions can comprise, e.g., zinc, nickel or cobalt, to which the His tag can bind with micromolar affinity. Suitable affinity media include Ni Sepharose, NTA-agarose, HisPur® resin (Thermo Scientific, Pierce Protein Products, Rockford, IL), or Talon® resin (Clontech, Mountain View, CA). The affinity matrix can then be washed with suitable buffers, e.g., phosphate buffers, to remove proteins that do not specifically interact with the cobalt or nickel ion. Washing efficiency can be improved by the addition of 20 mM imidazole. The biomolecule can optionally be eluted from the proteins are usually eluted with 150-300 mM imidazole). The purity and amount of purified biomolecule can then be assessed using suitable methods, e.g., SDS-PAGE and Western blotting.

[00264] Optionally, the His tag can be fused to a suitable amino acid sequence that facilitates removal of the His-tag using a suitable endopeptidase. Alternatively, the His tag may be removed using a suitable exopeptidase, for example the Qiagen TAGZyme exopeptidase.

[00265] In some embodiments, the His tag can facilitate linkage of the biomolecule to a metal surface, for example, a surface comprising Zn^{2+} , Ni^{2+} , Co^{2+} , or Cu^{2+} ions. Optionally, the His-tag can facilitate linkage of the biomolecule to the surface of a nanoparticle comprising one or more metal ions, typically via chelation interactions, as described in more detail herein.

[00266] Any suitable linkers can be used to link the biomolecules (including, e.g., the polymerases, nucleotides and nucleic acid molecules), the labels (including, e.g., nanoparticles, organic dyes, energy transfer moieties and/or other reporter moieties)

and/or the surfaces of the present disclosure to each other, in any combination. The linkers can be attached (to the surfaces, nanoparticles, polymerases, nucleotides, target nucleic acid molecules, primers, oligonucleotides, reporter moieties, and/or energy transfer moieties) via covalent bonding, non-covalent bonding, ionic bonding, hydrophobic interactions or any combination thereof. The type and length of the linker can be selected to optimize tethering, proximity, flexibility, rigidity, or orientation. The attachment can be reversible or non-reversible.

[00267] Suitable linkers include without limitation homobifunctional linkers and heterobifunctional linkers. For example, heterobifunctional linkers contain one end having a first reactive functionality to specifically link to a first molecule, and an opposite end having a second reactive functionality to specifically link to a second molecule. Depending on such factors as the molecules to be linked and the conditions in which the method of strand synthesis is performed, the linker can vary in length and composition for optimizing properties such as stability, length, FRET efficiency, resistance to certain chemicals and/or temperature parameters, and be of sufficient stereo-selectivity or size to link a label to the biomolecule such that the resultant conjugate is useful reporting biomolecular behavior such as approach, bonding, fusion or catalysis of a particular chemical reaction. Linkers can be employed using standard chemical techniques and include but not limited to, amine linkers for attaching labels to nucleotides (see, for example, U.S. Pat. No. 5,151,507); a linker containing a primary or secondary amine for linking a label to a nucleotide; and a rigid hydrocarbon arm added to a nucleotide base (see, for example, *Science* 282:1020-21, 1998).

[00268] In some embodiments, the linker comprises a polyethylene glycol (PEG) or PEG derivative. See, e.g., U.S. Provisional Applications 61/086,750; 61/102,709; 61/102,683; and 61/102,666. Such PEG moieties can be functionalized at one or both ends. In some embodiments, functionalization at both ends with the same reactive moiety can be employed to create a homobifunctional PEG derivative. Some examples of homobifunctional PEG derivatives include without limitation COOH-PEG-COOH; NH₂-PEG-NH₂; and MAL-PEG-MAL (where MAL denotes a maleimide group).

[00269] The linker moiety can optionally include: a covalent or non-covalent bond; amino acid tag; chemical compound (e.g., polyethylene glycol); protein-protein binding pair (e.g., biotin-avidin); affinity coupling; capture probes; or any combination of these.

[00270] Optionally, the linker can be selected such that it does not significantly interfere with the function or activity of the biomolecules, labels and/or surfaces that it links to each other. For example, when the biomolecule is a polymerase, the linker can be selected such that it does not significantly interfere with nucleotide binding to the polymerase, or with cleavage of the phosphodiester bonds, or with nucleotide incorporation, or with release of the polyphosphate product, or with translocation of the polymerase or with energy transfer, or with emission of a signal.

[00271] In some embodiments, the linker can comprise a single covalent bond or a series of covalent bonds. Optionally, the linker can be linear, branched, bifunctional, trifunctional, homofunctional, or heterofunctional. The linker can be cleavable. The linkers can be rigid or flexible. The linker can be capable of energy transfer. The linker can be a chemical chain or a chemical compound. The linker can be resistant to heat, salts, acids, bases, light and chemicals. The linker can include a short or long spacer, a hydrophilic spacer, or an extended spacer.

[00272] In another embodiment, a rigid linker can be used to link the biomolecule to the label. Examples of rigid linkers include benzyl linkers, proline or poly-proline linkers (S. Flemer, et al., 2008 *Journal Org. Chem.* 73:7593-7602), bis-azide linkers (M.P.L. Werts, et al., 2003 *Macromolecules* 36:7004-7013), and rigid linkers synthesized by modifying the so-called “click” chemistry scheme which is described by Megiatto and Schuster 2008 *Journal of the Am. Chem. Soc.* 130:12872-12873. In yet another embodiment, the linker can be an energy transfer linker synthesized using methods described in U.S. published patent application No. 2006/0057565, which is incorporated in its entirety. In yet another embodiment, the spacer linking moiety can be a cationic arginine spacer or an imidazolium spacer molecule.

[00273] In some embodiments, the linker moiety comprises about 1-40 plural valent atoms or more selected from the group consisting of C, N, O, S and P. The number of plural valent atoms in a linker may be, for example, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25, 30, or 40, or more. A linker may be linear or non-linear; some linkers have

pendant side chains or pendant functional groups (or both). Examples of such pendant moieties are hydrophilicity modifiers, for example solubilizing groups like, e.g., sulfo ($-\text{SO}_3\text{H}-$ or $-\text{SO}_3^-$). In some embodiments, a linker is composed of any combination of single, double, triple or aromatic carbon-carbon bonds, carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds and carbon-sulfur bonds. Exemplary linking members include a moiety which includes $-\text{C}(\text{O})\text{NH}-$, $-\text{C}(\text{O})\text{O}-$, $-\text{NH}-$, $-\text{S}-$, $-\text{O}-$, and the like. Linkers may by way of example consist of a combination of moieties selected from alkyl, alkylene, aryl, $-\text{C}(\text{O})\text{NH}-$, $-\text{C}(\text{O})\text{O}-$, $-\text{NH}-$, $-\text{S}-$, $-\text{O}-$, $-\text{C}(\text{O})-$, $-\text{S}(\text{O})_n-$ where n is 0, 1, 2, 3, 4, 5, or 6-membered monocyclic rings and optional pendant functional groups, for example sulfo, hydroxy and carboxy.

[00274] In some embodiments, the linker can result from “click” chemistries schemes (see, e.g., Gheorghe, et al., 2008 Organic Letters 10:4171-4174) which can be used to attach any combination of biomolecules, labels and surfaces as disclosed herein to each other

[00275] In one aspect, the linker can attach two or more energy transfer or reporter moieties to each other (the same type or different types of moieties).

[00276] In another aspect, a trifunctional linker (e.g., Graham, U.S. published patent application No. 2006/0003383) can be linked to two fluorescent dye moieties (the same type or different types) to amplify the fluorescent signal upon nucleotide binding or nucleotide incorporation. For example, a trifunctional linker can be linked to two energy transfer acceptor moieties, or to an energy transfer acceptor and a reporter moiety. In another example, multiple trifunctional linkers can be linked to each other, which can be linked to multiple fluorescent dyes for dendritic amplification of the fluorescent signal (e.g., Graham, U.S. published patent application No. 2007/0009980).

[00277] In some embodiments, the linker can be a cleavable linker such as, for example, a photocleavable linker, a chemically cleavable linker or a self-cleaving linker.

[00278] In some embodiments, the linker is a self-cleaving linker. Optionally, such linker can be a trimethyl lock or a quinone methide linker, which can each optionally link to two energy transfer acceptor and/or reporter moieties and the nucleotide.

[00279] In some embodiments, the linkers can be cleavable where cleavage is mediated by a chemical reaction, enzymatic activity, heat, acid, base, or light. For example, photo-cleavable linkers include nitrobenzyl derivatives, phenacyl groups, and benzoin esters. Many cleavable groups are known in the art and are commercially available. See, for example, J.W. Walker, et al., 1997 *Bioorg. Med. Chem. Lett.* 7:1243-1248; R. S. Givens, et al., 1997 *Journal of the American Chemical Society* 119:8369-8370; R. S. Givens, et al., 1997 *Journal of the American Chemical Society* 119:2453-2463; Jung et al., 1983 *Biochem. Biophys. Acta*, 761: 152-162; Joshi et al., 1990 *J. Biol. Chem.*, 265: 14518-14525; Zarling et al., 1980 *J. Immunol.*, 124: 913-920; Bouizar et al., 1986 *Eur. J. Biochem.*, 155: 141-147; Park et al., 1986 *J. Biol. Chem.*, 261: 205-210; and Browning et al., 1989 *J. Immunol.*, 143: 1859-1867; see also U.S. Patent No. 7,033,764. A broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) spacer arms with varying lengths are commercially available.

[00280] In yet another embodiment, the linker can be an energy transfer linker synthesized using methods described in U.S. Published Patent Application No. 2006/0057565.

[00281] In yet another embodiment, the linker can comprise a spacer, for example a cationic arginine spacer or an imidazolium spacer molecule.

[00282] In some embodiments, the linker can be a fragmentable linker, including non-lamellar "detergent-like" micelles or lamellar vesicle-like micelles such as small unilamellar vesicles or liposomes ("SUVs"), small multilamellar vesicles or liposomes (SMVs"), large unilamellar vesicles or liposomes ("LUVs") and/or large multilamellar vesicles or liposomes ("LMVs") (see U.S. Application No. 11/147827) and see U.S. Application Nos. 60/577995, and 12/188165.

[00283] In some embodiments, the linker can include multiple amino acid residues (e.g., arginine) which serve as an intervening linker between an attachment site on the biomolecule and the label. For example, the linker can be can four arginine residues which connect a dye moiety to a nucleotide comprising one or more phosphate groups, wherein the linker links the dye moiety to the terminal phosphate group of the nucleotide.

[00284] In some embodiments, linkers can be used to attach energy transfer or reporter moieties to biomolecules using any suitable linking procedure, including: amine linkers (see, for example, Hobbs, U.S. Pat. No. 5,151,507); a linker comprising a primary or secondary amine; and a rigid hydrocarbon arm (see, for example, R. F. Service, 1998 Science 282(5391):1020-21). Some exemplary linking procedures for attaching energy transfer or reporters moieties exemplary biomolecules are provided in European Patent Application 87310256.0; International Application PCT/US90/05565; Marshall, 1975 Histochemical Journal 7:299-303; and Barone et al., 2001 Nucleosides, Nucleotides, and Nucleic Acids, 20(4-7): 1141-1145. Other examples include linkers for attaching energy transfer or reporter moieties to exemplary biomolecules, using the specific example of oligonucleotides synthesized using phosphoramidate to incorporate amino-modified dT (see Mathies, U.S. Patent no. 5,707,804).

[00285] In one aspect, a linker comprising a polymer of ethylene oxide can be used to attach the surfaces, labels (including, e.g., dyes and nanoparticles), polymerases, nucleotides and/or nucleic acid molecules of the present disclosure to each other in any combination. Non-limiting examples of such polymers of ethylene oxide include polyethylene glycol (PEG), including short to very long PEG, branched PEG, amino-PEG-acids, PEG-amines, PEG-hydrazines, PEG-guanidines, PEG-azides, biotin-PEG, PEG-thiols, and PEG-maleinimides. For example, PEG includes: PEG-1000, PEG-2000, PEG-12-OMe, PEG-8-OH, PEG-12-COOH, and PEG-12-NH₂. In some embodiments, the PEG molecule may be linear or branched. In some embodiments, it can have a molecular weight greater than or approximately equal to 1000, 2000, 3000, 4000, 5000 or greater.

[00286] In some embodiments, functionalization with different reactive moieties can be used create a heterobifunctional PEG derivative comprising different reactive groups at each end. Such heterobifunctional PEGs can be useful in linking two entities, where a hydrophilic, flexible and biocompatible spacer is needed. Some examples of heterobifunctional PEG derivatives include without limitation Hydroxyl PEG Carboxyl (HO-PEG-COOH); Thiol PEG Carboxyl (HS-PEG-COOH); Hydroxyl PEG Amine (HO-PEG-NH₂); t-Boc Amine PEG Amine (TBOC-PEG-NH₂); Amine PEG Carboxyl (NH₂-PEG-COOH); t-Boc Amine PEG NHS Ester (TBOC-PEG-

NHS); Fmoc Amine PEG NHS Ester (Fmoc-PEG-NHS); Acrylate PEG NHS Ester (ACLT-PEG-NHS); Maleimide PEG Carboxyl (MAL-PEG-COOH); Maleimide PEG Amine (MAL-PEG-NH₂), including the TFA Salt thereof; Maleimide PEG NHS Ester (MAL-PEG-NHS); Biotin PEG NHS Ester (BIOTIN-PEG-NHS); Biotin Polyethylene Glycol Maleimide (BIOTIN-PEG-MAL); OPSS PEG NHS Ester (OPSS-PEG-NHS).

[00287] Optionally, the PEG derivative can be a multi-arm PEG derivative. In some embodiments, the multi-arm PEG derivative can be a PEG derivative having a core structure comprising pentaerythritol (including, for example, 4arm PEG Amine (4ARM-PEG-NH₂); 4arm PEG Carboxyl (4ARM-PEG-COOH); 4arm PEG Maleimide (4ARM-PEG-MAL); 4arm PEG Succinimidyl Succinate (4ARM-PEG-SS); 4arm PEG Succinimidyl Glutarate (4ARM-PEG-SG)); a PEG derivative having a core structure comprising hexaglycerin (including, for example, 8arm PEG Amine (8ARM-PEG-NH₂); 8arm PEG Carboxyl (8ARM-PEG-COOH); 8arm PEG Succinimidyl Succinate (8ARM-PEG-SS); 8arm PEG Amine (8ARM-PEG-SG); PEG derivative having a core structure comprising tripentaerythritol (including, for example, 8arm PEG Amine (8ARM(TP)-PEG-NH₂); 8arm PEG Carboxyl (8ARM(TP)-PEG-COOH); 8arm PEG Succinimidyl Succinate (8ARM(TP)-PEG-SS); 8arm PEG Amine (8ARM(TP)-PEG-SG)). Optionally, end groups for heterobifunctional PEGs can include maleimide, vinyl sulfones, pyridyl disulfide, amine, carboxylic acids and NHS esters. The activated PEG derivatives can then be used to attach the PEG to the desired biomolecule and/or label. Optionally, one or both ends of the PEG derivative can be attached to the N-terminal amino group or the C-terminal carboxylic acid of a protein-comprising biomolecule.

[00288] For methods, systems, compositions and kits comprising labeled biomolecule conjugates, the biomolecule can be linked to the label in any manner, and using any suitable linking procedures, that sufficiently preserves a particular biological activity of interest. Typically, when the biomolecule is a polymerase, the conjugate is a labeled polymerase conjugate and the biological activity of interest is polymerase activity. The polymerase of the labeled polymerase conjugate can be linked to the label using any suitable method that retains polymerase activity.

[00289] In some embodiments, the biomolecule and the label can be linked through a linker.

[00290] In some embodiments, the biomolecule-nanoparticle conjugate comprises a biomolecule covalently linked to a nanoparticle, wherein the biomolecule are linked to the nanoparticle through one or more covalent bonds.

[00291] In some embodiments, the label can be covalently linked to the biomolecule using any suitable method that permits linkage without loss of biological activity. Typically, the reagents employed are selected to allow the covalent linkage of the biomolecule to the label under defined reaction conditions. In some embodiments, the linkage can be performed in a site-specific manner.

[00292] In one exemplary embodiment, the label and the biomolecule can be reacted with each other in a suitable solvent in which both are soluble. The labels can optionally be treated or functionalized with suitable moieties to enhance their solubility in a suitable solvent.

[00293] In a typical embodiment, the biomolecule comprises a protein, more typically an enzyme, which can optionally be a polymerase. The various linking methods described herein have particular applicability for linking enzymes, e.g., polymerases, to labels such as nanoparticles or organic dyes.

[00294] In some embodiments, the label (e.g., nanoparticle or dye) can optionally be treated to create suitable sites for covalent attachment of the biomolecule. For example, the label and/or the biomolecule can be modified via introduction of a cysteine amino acid residue to create an attachment site comprising a free sulfhydryl group of the cysteine. In another embodiment, the label can be modified via introduction of a moiety comprising a reactive chemical group selected from any one of: a thiol group, an amino group (e.g., a primary or secondary amine) and a carboxyl group. The reactive chemical group of one member of the labeled biomolecule conjugate can then be reacted with a second reactive chemical group of a second member of the conjugate.

[00295] In one example, the label can be treated to introduce one or more reactive chemical groups that form suitable attachment sites for the biomolecule. Optionally, the label can be linked to with a cysteine-rich compound such as bovine serum albumin (BSA) or ovalbumin, resulting in the association of the cysteine-rich

compound with the label. The label can then be treated with reducing agents such as DTT, resulting in the formation of a large number of free sulfhydryl groups on the protein molecule to serve as potential sites for covalent attachment of the biomolecule. For example, reduction of a single BSA molecule on the nanoparticle surface results in the generation of approximately 34 free sulfhydryl groups, since a single BSA molecule typically comprises 17 disulfide bonds and one free thiol group. Such a poly-dentate thiol-modified protein could serve as a platform for initial modification of the label, onto which other biomolecules of interest can be added after the initial modification. In one embodiment, the BSA-treated label is a nanoparticle.

[00296] In some embodiments, the label can be treated or otherwise modified by the introduction of one or more reactive groups that can react with a second group on the biomolecule to link the label to the biomolecule.

[00297] In some embodiments, the label is a nanoparticle, and the reactive group is introduced via a “capping” process.

[00298] In one exemplary embodiment, the label can be modified by the introduction of one or more mercaptoacetic acid groups, and the resulting modified label can be covalently linked to the biomolecule through the acid group.

[00299] Optionally, the biomolecule can be engineered to include thiol-terminated residues that react with suitable groups on the label.

[00300] In another embodiment, the biomolecule and the label can be covalently linked through a condensation reaction between an amine of the biomolecule and a carboxyl group of the label using a suitable cross-linking agent. In some embodiments, the linker EDC is used to activate free –COOH ligands on the label. For example, the cross-linking agent 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) can be used to cross-link the amine-containing biomolecule with the carboxyl-containing label. The carboxyl group can be part of, or derived from, a mercaptoacetic acid or a dihydrolipoic acid (DHLA).

[00301] In some embodiments, the conjugation method can exploit the presence of cysteine residues within the biomolecule to be conjugated because such residues can serve as points of attachment to the label. For example, the thiol group of cysteine residues can be covalently linked to a label by using linking agents such as SMCC.

In some embodiments, the biomolecule is a polymerase that is genetically modified to introduce one or more cysteine residues placed in strategic positions, e.g., proximal to the active site/NTP binding pocket of the polypeptide. The polymerase can then be linked to a nanoparticle using SMCC. The covalent bond(s) between the polymerase and the label will not only stabilize the conjugate but also orient the polypeptide with respect to the nanoparticle in the preferred orientation for binding, resulting both in an increase of conjugate stability (manifested as reduced propensity of the conjugate to disassociate) and preservation of high affinity binding.

[00302] In some embodiments, the labeled biomolecule conjugate is produced by covalently linking the biomolecule to the label having one or more carboxyl groups using the heterobifunctional cross-linking agent succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate ("SMCC"). This agent comprises a maleimide reactive group capable of reacting with cysteine residues to form a thioether bond as well as an amine reactive NHS ester capable of reacting with primary amines to form an amide bond. Such a linker has utility in cross-linking, *inter alia*, biomolecules comprising one or more sulfhydryl groups (e.g., proteins comprising cysteine residues) to labels comprising one or more amine groups. Under suitable conditions, the double bond of the maleimide can undergo an alkylation reaction with a sulfhydryl group of the biomolecule to form a stable thioether bond. The NHS ester contains an amine-reactive group that can react with, *inter alia*, amine groups on the label. Optionally, the label includes a PEG amine, and the amine group that reacts with the NHS ester of SMCC can be the amine group of the PEG-amine.

In some embodiments, the labeled biomolecule conjugate comprises a biomolecule covalently linked to a label. In one exemplary embodiment, the biomolecule comprises one or more primary amine groups and the label is covalently linked to the one or more amine groups using the linking agents such as tris(hydroxymethyl)phosphine (TMP) and/or β -[tris(hydroxymethyl)phosphino] propionic acid (THPP). TMP and THPP are phosphine derivatives that can react with amines to form covalent linkages. See, e.g., Cochran, F., et al., "Application of tris(hydroxymethyl)phosphine as a coupling agent for alcohol dehydrogenase immobilization, *Enzyme & Microbial Technology* 18:373-378 (1996); Hermanson, G., *Bioconjugate Techniques*, Second Edition (2008).

[00303] In another exemplary embodiment, a lysine side chain of the biomolecule can be reacted with one or more labels comprising reactive NHS ester groups under conditions where the one or more labels bind to the one or more lysine side chains. The conditions can be selected such that one, few, some or all the lysine side chains are reacted with the NHS ester groups of the label. An exemplary embodiment wherein Cy3B comprising reactive NHS ester groups is reacted with Phi-29 polymerase to form a Cy3B-Phi-29 labeled polymerase conjugate comprising an average of one or two Cy3B dye molecules per polymerase is depicted in FIG. 3. Exemplary methods of preparing such conjugates are described in Examples 4 and 5, respectively.

[00304] In some embodiments, the conjugation method can exploit the presence of cysteine residues within the biomolecule to be conjugated because such residues can serve as points of attachment to the nanoparticle surface. For example, the thiol group of cysteine residues can be covalently linked to the surface of metal-containing nanoparticles by using linking agents such as SMCC. Such conjugation methods result in stable, oriented binding of the peptide and the nanoparticle. In some embodiments, the biomolecule is a protein or polypeptide, for example an enzyme, that comprises naturally occurring amino acid side chains that can be modified or otherwise treated so as to generate attachment sites for the nanoparticle. For example, the protein or polypeptide can be genetically modified to introduce one or more cysteine residues placed in strategic positions, e.g., proximal to the active site/NTP binding pocket of the polypeptide. The engineered polypeptide can then be linked to a nanoparticle using linkers such as SMCC. The covalent bond(s) between the polypeptide and the nanoparticle will not only stabilize the conjugate but also orient the polypeptide with respect to the nanoparticle in the preferred orientation for binding, resulting both in an increase of conjugate stability (manifested as reduced propensity of the conjugate to disassociate) and preservation of high affinity binding. The nanoparticle can optionally be derivatized with PEG-amine. In some embodiments, the nanoparticle can be capped with ovalbumin or other proteinaceous coating using any suitable cross-linkers (e.g., EDC, BS3 SMCC).

[00305] In some embodiments, nanoparticles containing free $-COOH$ ligands on their surface are derivatized via formation of amide bonds with the terminal amino

group of PEG-amine. The PEG-ylated nanoparticles are activated by treatment with agents such as SMCC, and the activated nanoparticles are then conjugated to polymerase via formation of thioether bonds involving the maleimide group of SMCC and a cysteine thiol group on the polymerase. In some embodiments, the linker EDC is used to activate free –COOH ligands on the surface of nanoparticles.

[00306] In some embodiments, the biomolecule is linked to the label via a covalent bond formed between a reactive α -thioester and an N-terminal cysteine residue. Such reactions are described, for example, in Dawson et al., *Science* 266:776-779 (1994); Dawson et al., *Ann. Rev. Biochem.* 69:923-960 (2000); Johnson et al., *JACS* 128:6640-6646 (2006). In some embodiments, a biomolecule that comprises, or is modified to comprise, an N-terminal cysteine group is linked to a label comprising a thioester ligand. In one example, a thioester-comprising crosslinker is first attached to the label using any suitable chemistry, resulting in a modified label comprising a reactive thioester. This modified label is then reacted with a biomolecule comprising, or modified to comprise, an N-terminal cysteine residue. Optionally, such reaction can be done in the presence of a suitable aromatic or aliphatic thiol catalyst. The thiol group of the cysteine reacts with the thioester on the label, forming a second thioester that undergoes intramolecular rearrangement. Such rearrangement results in the formation of a natural peptide bond linking the biomolecule to the label. *See, e.g.*, Dawson et al., *Science* 266:776-779 (1994); Dawson et al., *Ann. Rev. Biochem.* 69:923-960 (2000); Johnson et al., *JACS* 128:6640-6646 (2006).

[00307] In another exemplary embodiment, a label including a reactive aldehyde can be reacted with a biomolecule including an N-terminal cysteine residue. The reaction product is a five-membered thiazolidine ring that is stable over a pH range of 3-9. *See, e.g.*, Shao & Tam, “*Unprotected Peptides as Building Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone and Thiazolidine Linkages*,” *JACS* 117(14):3893-3899 (1995)). In some embodiments, the label includes an amine, which can be converted to an aldehyde through treatment with the heterobifunctional crosslinking reagent SFB (Pierce). The aldehyde of the label can then be reacted with an N-terminal cysteine group of a biomolecule to form a covalent linkage between the biomolecule and the label.

[00308] In yet another embodiment, a biomolecule comprising a ketone can be reacted with a label in the presence of a hydroxylamine to covalently link the biomolecule to the label.

[00309] In another exemplary embodiment, either the biomolecule or the label is modified to include an alkyne, and the other is modified to include an azide. The alkyne and azide can undergo a “click” reaction to form a covalent conjugate. Optionally, the click reaction can be a “copperless” click reaction.

[00310] In some embodiments, the biomolecule of the labeled biomolecule conjugate can be non-covalently linked to the label. See, for example, Goldman et al., 2005, *Anal. Chim. Acta* 534:63-67. For example, in some embodiments the biomolecule can be linked to the label via a non-covalent interaction between a first and second member of a binding pair, as described further herein.

[00311] In some embodiments, the conjugation of the biomolecule to the label can be achieved through a process of self-assembly, wherein suitably modified biomolecules and labels are contacted under conditions where they will bind spontaneously to each other. For example, one or more thiolated proteins can be conjugated to a sulfur-comprising label using dative thiol-bonding between the cysteine residues on the protein and a sulfur atom of the label. The label can be a nanoparticle. See, e.g., Akerman, M. E., et al., “Nanocrystal targeting in vivo”, *Proc. Natl. Acad. Sci. USA* 99:12617-12621 (2002). Optionally, the conjugate can be formed through adsorption or non-covalent self-assembly of proteins with the label.

[00312] In some embodiments, the conjugate can be formed through self-assembly via electrostatic interactions, wherein biomolecules having either a natural positive surface charge or that are engineered to include positively charged domains, interact with labels having a negative surface charge, (e.g., nanoparticles capped with substances comprising COOH moieties). See, for example, Mattoussi et al., “Self-assembly of CdSe-ZnS nanoparticle bioconjugates using an engineered recombinant protein”, *J. Am. Chem. Soc.* 122(49):12142-50 (2000); Mattoussi et al., “Bioconjugation of highly luminescent colloidal CdSe-ZnS nanoparticles with an engineered two-domain recombinant protein”, *Phys. Status Solido B-Basic Res.* 224:277-83 (2001). In some embodiments, the label can be modified via the introduction of one or more -COOH ligands or other negatively charged moieties (for

example, lipoic acid moieties), and then contacted with engineered recombinant protein-comprising biomolecules comprising positively charged attachment domains (for example, leucine zippers, polylysine or polyarginine linkers or the like).

[00313] Another assembly-based approach involves the use of affinity ligands. In some embodiments, conjugation is accomplished through use of binding pairs.

[00314] Provided herein are labeled biomolecule conjugates comprising a biomolecule linked to a label by means of a binding pair. In one example, a first member of a binding pair is linked to a biomolecule and a second member of the binding pair is linked to the label. The first and second members of the binding pair are linked to each other, thereby linking the biomolecule to the label.

[00315] Also provided herein are methods for making a labeled biomolecule conjugate, comprising: linking a first member of a binding pair to a biomolecule; linking a second member of the binding pair to at least one label; and contacting the first and second members under conditions where they bind to each other, thereby linking the biomolecule to the at least one label.

[00316] Optionally, the biomolecule can be an enzyme, for example a polymerase. In some embodiments, the first member of the binding pair is a biotin moiety and the second member is an avidin moiety. In some embodiments, the second member of the binding pair is linked to three, four, five, six, seven or more labels.

[00317] In some embodiments, the biomolecule is a polymerase and the conjugate is a labeled polymerase conjugate. In some embodiments, the polymerase of the conjugate can retain at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% polymerase activity relative to the unconjugated form of the polymerase. Typically, the polymerase activity can be measured using any one of the primer extension activity assays described herein.

[00318] Suitable binding pairs include: a biotin moiety (including, for example, biotin, desthiobiotin or photoactivatable biotin, bound with an avidin moiety, such as streptavidin or neutravidin); His-tag bound with nickel or cobalt; maltose bound with a maltose binding protein (MBP); lectin bound with a carbohydrate; calcium bound with a calcium binding protein (CBP); antigen or epitope tags bound with an antibody or antibody fragment; particular antigens such as digoxigenin, fluorescein, nitrophenol or bromodeoxyuridine and their respective antibodies; IgG bound with

protein A; receptor bound with a receptor agonist or antagonist; enzyme bound with an enzyme cofactors; and thyroxine bound with cortisol.

[00319] In some embodiments, the members of a binding pair can be naturally occurring substances or else substances which are prepared, for example, by means of chemical synthesis, microbiological techniques and/or recombinant DNA methods.

[00320] In some embodiments, the binding pair members can be selected and/or identified using phage display libraries, synthetic peptide databases or recombinatorial antibody libraries. See, e.g., Larrick & Fry (1991) *Human Antibodies and Hybridomas*, 2: 172-189.

[00321] In one exemplary embodiment, the biomolecule and the label are linked by means of a binding pair comprising a biotin moiety and an avidin moiety. The strong interaction between streptavidin (or avidin) and biotin (cis-hexahydro-2-oxo-1H-thieno[3,4]imidazole-4-pentanoic acid) is well known. The affinity binding between streptavidin and biotin, having a dissociation constant, K_d , of approximately 10^{-15} M, is regarded as one of the strongest known, non-covalent, biochemical interactions. The biotin-avidin bond forms very rapidly and is considered to be stable under a wide range of pH, temperature and other denaturing conditions. See, e.g., Savage et al., *Avidin-Biotin Chemistry: A Handbook*, 1992:1-23, Rockford, Pierce Chemical Company; Goldman et al., "Avidin: A natural bridge for quantum dot-antibody conjugates", *J. Am. Chem. Soc.* 124:6378-6382 (2002). Without being bound by any particular theory, it is believed that biotin and avidin moieties bond with each other through a combination of ionic (electrostatic) and hydrophobic interactions.

[00322] The avidin moiety can in some embodiments comprise any suitable naturally occurring avidin, as well as non-naturally occurring derivatives and analogs thereof. Some of these materials are commercially available, e.g. native avidin and streptavidin, nonglycosylated avidins, N-acyl avidins and truncated streptavidin, or can be prepared by well-known methods (see Green, 1990, for preparation of avidin and streptavidin; Hiller et al., 1990, for preparation of non-glycosylated avidin; Bayer et al., 1990, for the preparation of streptavidin and truncated streptavidin).

[00323] In an exemplary embodiment, a biomolecule and a label can be linked to form a labeled biomolecule conjugate, where the linkage between the biomolecule and the label comprises one or more affinity interactions between a biotin moiety and

an avidin moiety. In some embodiments, a biomolecule can be linked to a biotin moiety and a label can be linked to an avidin moiety, where the biomolecule is linked to the label via an affinity interaction between the biotin and avidin moieties. The label can comprise, for example, an organic dye moiety or a nanoparticle.

[00324] In some embodiments, a biotin moiety is linked to either the biomolecule or the label through treatment with an enzyme that is capable of covalently attaching a biotin moiety to a substrate, such as a biotin ligase. For example, the biotin ligase can be the *E. coli* biotin ligase (EC 6.3.4.15) encoded by the *birA* gene of *E. coli*. The *E. coli* biotin ligase is also commonly referred to as biotin-protein ligase; other names for this enzyme include: biotin ligase; biotin operon repressor protein; *birA*; biotin holoenzyme synthetase; biotin-[acetyl-CoA carboxylase] synthetase. This enzyme can activate a biotin moiety to form a biotinyl-5' adenylate and can transfer the activated biotin moiety to a biotin-accepting protein, such as an acceptor peptide for biotin ligase (hereinafter, "a biotin acceptor peptide"). In some embodiments, the biotin acceptor site can comprise the amino acid sequence of SEQ ID NO: 10: See, e.g., Howarth et al., "Targeting quantum dots to surface proteins in living cells with biotin ligase", *Proc. Natl. Acad. Sci. USA* 102(21):7583-7588 (2005). In some embodiments, the biotin acceptor peptide can comprise the amino acid sequence of SEQ ID NO: 10 (GLNDIFEAQKIEWHE). Optionally, the biotin acceptor peptide is the AviTag™ peptide (Avidity, LLC). See, e.g., U.S. Patent Nos. 5,723,584, 5,874,239 and 5,932,433.

[00325] In some embodiments, the biotin can be linked to a thiol group of the biomolecule. For example, the biomolecule can comprise a free cysteine residue (including but not limited to a naturally occurring or an engineered replacement cysteine residue), and the biotin moiety can be linked to the free cysteine residue. Optionally, the biotin moiety can be linked to the cysteine residue by use of a thiol-reactive reagent, such as a biotin-maleimide reagent, to form a biotin-labeled biomolecule. See, e.g., U.S. Patent No. 7,521,541.

[00326] In one exemplary embodiment, the label of the conjugate is linked to an avidin moiety and contacted with a biomolecule linked to a biotin moiety.

[00327] In some embodiments, the label is linked to the biomolecule through use of an attachment site that is engineered or otherwise introduced into the biomolecule and

serves as the site of attachment for one or more labels. In some embodiments, the introduced attachment site comprises an enzyme modification recognition sequence. In some embodiments, the modification enzyme recognition sequence can comprise a biotin ligase acceptor site, to which one or more biotin moieties can be attached by a biotin ligase, thus forming an attachment site for a label linked to an avidin moiety.

[00328] In some embodiments, the biomolecule of the conjugate is a protein that comprises a biotin ligase acceptor site, such that the biomolecule can be biotinylated via treatment with a suitable biotin ligase in the presence of a biotin. Typically, the biomolecule or the nanoparticle to be biotinylated comprises a biotin acceptor site.

[00329] In another embodiment, the modification enzyme recognition site is an N-terminal recognition site for the TEV (Tobacco Etch Virus) protease enzyme. Typically, such recognition site comprises the following amino acid sequence:

[00330] ENLYFQ SEQ ID NO: 37

[00331] The TEV protease can specifically cleave this modification enzyme recognition sequence after the glutamine (Q) residue. See, e.g., de Graaf et al., “*Nonnatural Amino Acids for Site-Specific Protein Conjugation*,” 20(7):1281-1295 (2009); Tolbert & Wong, “Conjugation of Glycopeptide Thioesters To Expressed Protein Fragments”, *Methods in Mol. Bio.*, Vol. 283 (“Bioconjugation Protocols”), pp.255-266 (2004). Optionally the protein to be conjugated is fused at its N-terminus with a peptide tag comprising the TEV protease recognition sequence, and the recombinant protein is then cleaved with TEV protease to remove the tag and uncover an N-terminal cysteine. The amino acid sequences of exemplary polymerases comprising a TEV protease recognition sequence at their N-terminus are disclosed herein. Biomolecules comprising a N-terminal cysteine are especially desirable because the N-terminal cysteine group can serve as an attachment site for the site-specific attachment of a label using a variety of different chemistries. In one example, the N-terminal cysteine can be reacted with a thioester to form a peptide bond (see FIG. 17).

[00332] In another embodiment, the N-terminal cysteine can be reacted with an aldehyde to form a 5-membered thiazolidine ring (see FIG. 18). Specific examples using such conjugation strategies are described further herein.

[00333] In some embodiments, the enzyme can be labeled in a site-specific manner through incorporation of unnatural or modified amino acids during translation of the mRNA encoding the enzyme using modified aminoacyl tRNAs and/or modified tRNA synthetases. Briefly, such unnatural amino acids are genetically encoded in mammalian and other cells by using a mutant *E. coli* aminoacyl-tRNA synthetase that has been evolved to selectively aminoacylate its tRNA with the unnatural amino acid of interest. This mutant synthetase, together with an amber suppressor tRNA, can be used to selectively incorporate the unnatural amino acid into a protein at selected sites in response to amber nonsense codes. See, e.g., Brustad et al., "A general and efficient method for the site-specific dual-labeling of proteins for single molecule fluorescence resonance energy transfer" *J. Am. Chem. Soc.* 130(52):17664-5 (2008); Liu et al., "Genetic incorporation of unnatural amino acids into proteins in mammalian cells" *Nat. Methods*, 4(3):239-244 (2007).

[00334] In another covalent conjugation approaches can also involve the insertion of other non-natural amino acids besides an N-terminal cysteine at defined sites within the biomolecule via site-specific engineering. Such introduced non-natural amino acids can then serve as attachment sites for a label. In one exemplary embodiment, an azido non-natural amino acid is engineered into the polymerase, which is then undergoes a click chemistry reaction with a DIBO moiety (DIBO being a reactive "click" complement to the azide). The DIBO moiety can optionally be linked to the termini of PEG surface ligands of a nanoparticle. In another embodiment, a heterobifunctional crosslinker can be used; one end of the linker can include a His-tag for metal affinity binding to the nanoparticle surface, and the other end can include a DIBO moiety for covalent coupling to the azido non-natural amino acid. The linker can optionally comprise peptide or PEG chains. Use of such a heterobifunctional crosslinker eliminates the need to develop nanoparticles with specific covalent chemistry.

[00335] In another embodiment, a ketone-comprising non-natural amino acid is introduced into a biomolecule, which can serve as an attachment site for a nanoparticle. For example, the introduced ketone group can allow site-specific modification of the biomolecule using chemistry unique to the ketone functional group. FIG. 19 depicts one exemplary pathway by which the insertion of the

nonnatural amino acid, acetylphenyl alanine, into an exemplary biomolecule (Phi29 DNA polymerase) can be used to covalently couple a nanoparticle to the biomolecule. [00336] In another embodiment, an azide-comprising non-natural amino acid such as, for example, azidophenyl alanine, can be introduced into a biomolecule; the introduced azide can then be used in a “click” reaction to covalently couple the biomolecule to an alkyne-comprising biomolecule. Normally, alkyne-azide click reactions utilize copper as a catalyst. In some embodiments, a “copperless click” reaction is utilized where a strained, eight-membered ring containing an alkyne can undergo reaction with an azide without the use of a copper catalyst. FIG. 20 depicts one exemplary pathway by which the insertion of the nonnatural amino acid, azidophenyl alanine, into an exemplary biomolecule (Phi29 DNA polymerase) can be used to covalently couple a nanoparticle to the biomolecule.

[00337] In some embodiments, the conjugate can be formed through self-assembly of the biomolecule with the label, where the self-assembly involves the formation of one or more metal affinity-based interactions, a phenomenon also referred to herein as “chelation”. In some embodiments, one or more polypeptides can be conjugated to a label through metal-affinity coordination between a histidine residue of the polypeptide and a metal atom of the label. This strong interaction (Zn^{2+} -His) has a dissociation constant, K_D , stronger than most antigen-antibody bindings (10^6 - 10^9). See, e.g., Hainfeld et al., “Ni-NTA-gold clusters target his-tagged proteins”, J. Struct. Biol. 127:185-198 (1999). Nanoparticles are one example of a label that comprises metal atoms that are accessible for binding by histidine residues of a biomolecule.

[00338] In some embodiments, the biomolecule can be engineered to contain, or otherwise fused to, a genetically encoded domain that exhibits chelation-based interactions with a metal-containing label. See, for example, Clapp et al., Nature Protocols 1(3):1258 (2006). In some embodiments, such domain can comprise one or more His tags. For example, the His tagged biomolecules, e.g., proteins, can bind via metal affinity-based interactions to labels comprising lipoic acid or other negatively charged moieties. It is theorized that the strength of such binding is determined by the degree to which the imidazole side chains of the oligohistidine segment of the His tag interact with the metal ions of the label. Without being bound to any particular theory or mechanism for such linkage, such methods are within the scope and spirit of

the present disclosure. Overall, such methods can simplify the bioconjugation procedure and reduce the overall hydrodynamic size of the resulting conjugate by eliminating the need for a bridging protein. Such preparation methods can be particularly suitable for FRET applications that require reduced spacing between the donor and acceptor moieties. The bioconjugate size can be further reduced by using only the shorter polymerase fragments that eliminate regions not required for core polymerase function.

[00339] In some embodiments, the biomolecule, e.g., protein, comprises one or more consecutive histidine residues, linked to the label. In one exemplary embodiment, the biomolecule comprises between four and twelve consecutive histidine residues.

[00340] In some embodiments, the label can be linked to a chelating compound, e.g., nickel-nitriloacetic acid, Ni-NTA) that quantitatively binds to His-tagged biomolecules with controlled molar ratio and biomolecular orientation. The K_D for the hexahistidine tag (His₆) and Ni-NTA is 10^{-13} .

[00341] In some embodiments, the conjugate comprises a polymerase fused to a His-tag and linked to a label. The His-tag can comprise 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more consecutive Histidine residues. The His-tag can be conjugated to the N-terminus of the polymerase, the C-terminus of the polymerase, or to any other suitable site within the polymerase. In some embodiments, a His-tag comprising six consecutive histidine residues is fused to the N-terminus of the polymerase. Optionally, the His-tag and the polymerase open reading frame can be separated by a peptide linker sequence, which can comprise the F-linker or H-linker sequence.

[00342] In some embodiments, the F-linker is situated between an N-terminal His-tag comprising six consecutive histidine residues and the protein.

[00343] Also disclosed herein are compositions relating to multiply labeled biomolecule conjugates comprising a biomolecule linked to a plurality of labels, wherein the conjugate has a biological activity that is characteristic of the biomolecule. The plurality of labels can comprise two, three, four, five, six, seven or more labels. Also disclosed herein are methods of making labeled biomolecule conjugates comprising a biomolecule linked to a plurality of labels, as well as methods, systems and kits for making and using such conjugates.

[00344] Such labeled biomolecule conjugates comprising a polymerase linked to two, three, four, five, six, seven or more labels and having polymerase activity can provide a range of benefits. For example, such compositions can be provide several advantages in FRET based assays. In some embodiments, such conjugates can be used in conjunction with shorter excitation wavelengths, resulting in reduced direct excitation of the acceptor dye (false positive signal), and/or reduced donor emission bleed-through into the acceptor emission channel. A higher signal-to-noise ratio can thereby be achieved.

[00345] In some embodiments, the multiply labeled biomolecule conjugate can include a biomolecule (e.g., polymerase) linked to a first member of the binding pair, and a second member of a binding pair linked to a plurality of labels, wherein the first member and second member of the binding pair are linked to each other, thereby linking the biomolecule to the plurality of labels. In some embodiments, the binding pair comprises an avidin moiety and a biotin moiety, but in more general terms such dual labeled constructs can be generated using a variety of attachment moieties, wherein the attachment moiety serves to link the polymerase to two or more labels.

[00346] Attachment Moiety

[00347] Also disclosed herein are labeled enzyme conjugates, comprising: an enzyme linked to an attachment moiety, wherein the attachment moiety is linked to one or more labels, thereby linking the enzyme to the label to form a labeled enzyme conjugate. In some embodiments, the attachment moiety can be linked to at least three, four, five, six, seven or more labels.

[00348] The attachment moiety can be any suitable moiety, molecule or structure that serves to link one or more labels to the enzyme. In some embodiments, the attachment moiety can have reactive sites or groups, which can facilitate the formation of a linkage between the attachment moiety and the enzyme on the one hand, and/or between the attachment moiety and the one or more labels on the other. In some embodiments, the attachment moiety comprises one, two, three, four or more reactive sites that can facilitate linkage of the attachment moiety with an enzyme and/or labels. In some embodiments, one, some or all of the reactive sites of the attachment moiety can spontaneously bind to the enzyme and/or the one or more labels, thereby creating a linkage between the attachment moiety and the enzyme

and/or the one or more labels. In other embodiments, the reactive site may need to undergo activation or other suitable treatment before it can bind to the enzyme and/or the one or more labels.

[00349] In some embodiments, the attachment moiety comprises an avidin moiety. In some embodiments, the avidin moiety comprises one, two three, four or more reactive sites capable of spontaneously binding to the biotin moiety of a biotinylated label. In some embodiments, the enzyme is a polymerase, the attachment moiety is linked to a polymerase, and the attachment moiety further comprises an avidin moiety linked to four biotinylated labels, thereby forming a polymerase linked to four labels through the attachment moiety.

[00350] In some embodiments, the attachment moiety can be linked to two or more different types of labels. Optionally, the attachment moiety can be further linked to a biomolecule, which in some embodiments can be a polymerase. An illustrative non-limiting example of a labeled polymerase conjugate, comprising a polymerase linked to an attachment moiety, wherein the attachment moiety is linked to two different types of labels, is depicted in FIG. 2. (In FIG. 2, the attachment moiety is referred to as a “bridging molecule”, but this phrase is in no way intended to limit the attachment moiety in terms of function or structure). Preparation of an exemplary labeled polymerase conjugate comprising a polymerase linked to two different kinds of labels using an attachment moiety is described in Example 3, below.

[00351] Any suitable method capable of generating conjugates suitable for use in the desired applications, as well as by combination of such techniques, both covalent and non-covalent, may be employed to form conjugates according to the present disclosure. For example, a conjugate may be formed through a combination of both electrostatic and affinity-based interactions. In some embodiments, the polymerase can be engineered to include attachment domains that can mediate a range of interactions with the nanoparticle surface. For example, the polymerase can be fused both to a polyhistidine tag as well as a leucine zipper. Alternatively, the polymerase can be fused to a single attachment domain comprising histidines interspersed with, or flanked by, several lysine and/or arginine residues. Advantages of using such “multifunctional” attachment domains include increased strength of binding, the

ability to orient binding between the polymerase and nanoparticle, and the ability to perform conjugations at ultra-high dilutions.

[00352] In addition to assembling the conjugate via direct interactions between the tagged peptide and metal atoms (or metal ions) present in the label, another option is to attach or cross-link ligands derivatized distally with species capable of interacting with and immobilizing a suitably modified peptide. A chelator molecule such as nitrilotriacetic acid (NTA) can be attached covalently to the label. This can be achieved by carbodiimide mediated coupling of one of the acetic acid side chains of NTA to a functional group of the label such as an amine. The resulting product can then be contacted with a solution of metal ions, such as Ni^{2+} , allowing some of the latter to bind to the chelating functional groups of NTA. After removal of the excess non-chelated metal ions, the metal-derivatized labels can be contacted with a solution of a His tag modified biomolecule, whereby the histidine residues of the poly-His tag will form additional coordinative chemical bonds with the NTA-chelated Ni^{2+} ions. As result, the His-tagged biomolecule will be immobilized to the label. This technique results in strong, oriented binding between the peptide and the nanoparticle, with only minor sacrifices in FRET R_0 distance compared to direct binding of the His tag to the nanoparticle surface, and creates a conjugate with a more fully protected surface that better withstands environmental stresses.

[00353] One general advantage of all assembly-based methods is that the need for linkers and/or cross-linking treatments is obviated, resulting in much greater simplicity and ease of synthesis. Unlike covalent cross-linking techniques, self-assembly of fusion peptides tagged with an attachment domain, such as a His tag, with labels eliminates the requirement for developing specific chemical synthetic routes for each label on a case-by-case basis. Another advantage of self-assembly based methods is the ability to selectively engineer the properties of the attachment domain, e.g., size, charge, and pH or temperature stability so as to control its binding properties. This also allows control of the assembly of individual peptides, e.g., into monomers, dimers, trimers, tetramers, etc., ultimately allowing control of the protein packing around the nanoparticles to form complex bioconjugates.

[00354] In some embodiments, the conjugate can be formed through a combination of methods, including self-assembly and covalent attachment. For example, the metal

affinity- or chelation-based binding of fusion proteins to the label can be reinforced by subsequent treatment to induce the formation of covalent bonds between the polymerase and the label. In one exemplary embodiment a photoreactive crosslinker, such as substituted arylazide, diazirine or benzophenone derivatives, can be attached to the label, which is subsequently contacted with a His-tagged polymerase. Following affinity-based assembly of the polymerase and label, the assembled complex can be photoirradiated to generate a covalently linked conjugate via crosslinking.

[00355] Further provided herein is a composition comprising a population of labeled biomolecule conjugates, wherein an average of at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97% or 99% of the population include three or more labels linked to the biomolecule.

[00356] Further provided herein is a composition comprising a population of labeled biomolecule conjugates, wherein an average of at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97% or 99% of the population include four or more labels linked to the biomolecule.

[00357] Further provided herein is a composition comprising a population of labeled biomolecule conjugates, wherein an average of at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97% or 99% of the population include five or more labels linked to the biomolecule.

[00358] Further provided herein is a composition comprising a population of labeled biomolecule conjugates, wherein an average of at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97% or 99% of the population include six or more labels linked to the biomolecule.

[00359] In some embodiments, provided herein is a population of labeled biomolecule conjugates wherein an average of at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, or 99% of the biomolecule-nanoparticle conjugates in the population comprise an average of about one biomolecule and about one label.

[00360] In some embodiments, provided herein is a population of labeled biomolecule conjugates where an average of at least 30%, at least 40%, at least 50%, at least 60% at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at

least 98%, at least 99% or more of conjugates in the population comprise an average of about one biomolecule per conjugate.

[00361] Also disclosed herein is a population of conjugates, each conjugate comprising a biomolecule linked to a label, and the population comprising an average of between 0.5-1.5 biomolecules per label.

[00362] Also disclosed herein is a method for preparing a 1:2 biomolecule:label conjugate using linker moieties. For example, a label dimer linked by a short organic molecule, for example, 4,4'-biphenyldithiol, can be attached to the biomolecule via a linker attached to any suitable moiety, e.g., a phenyl group, within the dimer linker. For example, the biomolecule could first be attached to the linker molecule using another linking moiety, and then each end of the linker could be attached to a label. Dimers could be purified via filtration or other suitable techniques.

[00363] In some embodiments, the label can be conjugated both to a biomolecule as well as other proteins, especially proteins known to enhance biomolecular activity or have other beneficial side effects. For example, the label can optionally be conjugated both to a polymerase and to Single-Stranded DNA Binding Protein (SSBP), various processivity factors such as LEF-3, or the herpes simplex virus UL42 protein. The presence of such proteins can help to reduce the number of biomolecules linked to the label and at the same time stabilize the conjugate complex, resulting in enhancement of DNA synthesis and increased read lengths. Alternatively, proteins that reduce the potential photodamage caused by reactive oxygen species, such as catalase or superoxide dismutase (SOD), can also be conjugated to the label in combination with the biomolecule.

[00364] The labeled biomolecule conjugates of the present disclosure can optionally comprise any polymerase suitable for use in the particular biological application of interest.

[00365] In some embodiments, the compositions, methods, systems and kits of the present disclosure relate to labeled biomolecule conjugates comprising a polymerase. In some embodiments, the polymerase incorporates one or more nucleotides into a nucleic acid molecule and the resulting one or more nucleotide incorporations is detected and/or analyzed in real time.

[00366] In some embodiments, the polymerase can bind a target nucleic acid molecule, which may or may not be base-paired with a polymerization initiation site (e.g., primer).

[00367] Typically, the polymerase can selectively bind to a nucleotide. Such nucleotide binding can occur in a template-dependent or non-template-dependent manner. Typically, the polymerase can mediate cleavage of the bound nucleotide. Typically, such cleavage of the nucleotide results in the formation of at least two nucleotide cleavage products. For polyphosphate-comprising nucleotides, such cleavage will typically occur between the α and β phosphate groups. Typically, the polymerase can mediate incorporation of one of the nucleotide cleavage products into a nucleic acid molecule, and release of another nucleotide cleavage product. When used in conjunction with polyphosphate-comprising nucleotides, the released nucleotide cleavage product can comprise one or more phosphates (for example, a polyphosphate chain); for nucleotides that are non-phosphate-comprising analogs, the nucleotide cleavage product may not comprise any phosphorus.

[00368] In some embodiments, the polymerase can mediate incorporation of a nucleotide on to a polymerization initiation site (e.g., terminal 3'OH of a primer).

[00369] In some embodiments, the polymerase can be unlabeled. Alternatively, the polymerase can be linked to one or more label. In some embodiments, the label comprises at least one energy transfer moiety.

[00370] The polymerase may be linked with at least one energy transfer donor moiety. One or more energy transfer donor moieties can be linked to the polymerase at the amino end or carboxyl end or may be inserted at any site therebetween. Optionally, the energy transfer donor moiety can be attached to the polymerase in a manner which does not significantly interfere with the nucleotide binding activity, or with the nucleotide incorporation activity of the polymerase. In such embodiments, the energy transfer moiety is attached to the polymerase in a manner that does not significantly interfere with polymerase activity.

[00371] In one aspect, a single energy transfer donor moiety can be linked to more than one polymerase and the attachment can be at the amino end or carboxyl end or may be inserted within the polymerase.

[00372] In another aspect, a single energy transfer donor moiety can be linked to one polymerase.

[00373] In one aspect, the energy transfer donor moiety can be a nanoparticle (e.g., a fluorescent nanoparticle) or a fluorescent dye. The polymerase, which can be linked to the nanoparticle or fluorescent dye, typically retains one or more activities that are characteristic of the polymerase, e.g., polymerase activity, exonuclease activity, nucleotide binding, and the like.

[00374] In one aspect, the polymerases can be replicases, DNA-dependent polymerases, primases, RNA-dependent polymerases (including RNA-dependent DNA polymerases such as, for example, reverse transcriptases), strand-displacement polymerases, or thermo-stable polymerases. In another aspect, the polymerase can be any Family A or B type polymerase. Many types of Family A (e.g., *E. coli* Pol I), B (e.g., *E. coli* Pol II), C (e.g., *E. coli* Pol III), D (e.g., Euryarchaeotic Pol II), X (e.g., human Pol beta), and Y (e.g., *E. coli* UmuC/DinB and eukaryotic RAD30/xeroderma pigmentosum variants) polymerases are described in Rothwell and Watsman 2005 *Advances in Protein Chemistry* 71:401-440.

[00375] In yet another aspect, the polymerases can be isolated from a cell, or generated using recombinant DNA technology or chemical synthesis methods. In another aspect, the polymerases can be expressed in prokaryote, eukaryote, viral, or phage organisms. In another aspect, the polymerases can be post-translationally modified proteins or fragments thereof.

[00376] In one aspect, the polymerase can be a recombinant protein which is produced by a suitable expression vector/host cell system. The polymerases can be encoded by suitable recombinant expression vectors carrying inserted nucleotide sequences of the polymerases. The polymerase sequence can be linked to a suitable expression vector. The polymerase sequence can be inserted in-frame into the suitable expression vector. The suitable expression vector can replicate in a phage host, or a prokaryotic or eukaryotic host cell. The suitable expression vector can replicate autonomously in the host cell, or can be inserted into the host cell's genome and be replicated as part of the host genome. The suitable expression vector can carry a selectable marker which confers resistance to drugs (e.g., kanamycin, ampicillin, tetracycline, chloramphenicol, or the like), or confers a nutrient requirement. The

suitable expression vector can have one or more restriction sites for inserting the nucleic acid molecule of interest. The suitable expression vector can include expression control sequences for regulating transcription and/or translation of the encoded sequence. The expression control sequences can include: promoters (e.g., inducible or constitutive), enhancers, transcription terminators, and secretion signals. The expression vector can be a plasmid, cosmid, or phage vector. The expression vector can enter a host cell which can replicate the vector, produce an RNA transcript of the inserted sequence, and/or produce protein encoded by the inserted sequence. The recombinant polymerase can include an affinity tag for enrichment or purification, including a poly-amino acid tag (e.g., poly His tag), GST, and/or HA sequence tag. Methods for preparing suitable recombinant expression vectors and expressing the RNA and/or protein encoded by the inserted sequences are well known (Sambrook et al, *Molecular Cloning* (1989)).

[00377] The polymerases may be DNA polymerases and include without limitation bacterial DNA polymerases, prokaryotic DNA polymerase, eukaryotic DNA polymerases, archaeal DNA polymerases, viral DNA polymerases and phage DNA polymerases. The polymerase can be a commercially available polymerase.

[00378] In some embodiments, the polymerase can be a DNA polymerase and include without limitation bacterial DNA polymerases, eukaryotic DNA polymerases, archaeal DNA polymerases, viral DNA polymerases and phage DNA polymerases.

[00379] Suitable bacterial DNA polymerase include without limitation *E. coli* DNA polymerases I, II and III, IV and V, the Klenow fragment of *E. coli* DNA polymerase, *Clostridium stercorarium* (Cst) DNA polymerase, *Clostridium thermocellum* (Cth) DNA polymerase and *Sulfolobus solfataricus* (Sso) DNA polymerase.

[00380] Suitable eukaryotic DNA polymerases include without limitation the DNA polymerases α , δ , ϵ , η , ζ , γ , β , σ , λ , μ , ι , and κ , as well as the Rev1 polymerase (terminal deoxycytidyl transferase) and terminal deoxynucleotidyl transferase (TdT).

[00381] Suitable viral and/or phage DNA polymerases include without limitation T4 DNA polymerase, T5 DNA polymerase, T7 DNA polymerase, Phi-15 DNA polymerase, Phi-29 DNA polymerase (see, e.g., U.S. Patent No. 5,198,543; also referred to variously as Φ 29 polymerase, phi29 polymerase, phi 29 polymerase, Phi 29 polymerase, and Phi29 polymerase); Φ 15 polymerase (also referred to herein as

Phi-15 polymerase); Φ 21 polymerase (Phi-21 polymerase); PZA polymerase; PZE polymerase, PRD1 polymerase; Nf polymerase; M2Y polymerase; SF5 polymerase; f1 DNA polymerase, Cp-1 polymerase; Cp-5 polymerase; Cp-7 polymerase; PR4 polymerase; PR5 polymerase; PR722 polymerase; L17 polymerase; M13 DNA polymerase, RB69 DNA polymerase, G1 polymerase; GA-1 polymerase, BS32 polymerase; B103 polymerase; BA103 polymerase, a polymerase obtained from any phi-29 like phage or derivatives thereof, etc. See, e.g., U.S. Patent No. 5,576,204, filed Feb. 11, 1993; U.S. Pat. Appl. No. 2007/0196846, published Aug. 23, 2007.

[00382] Suitable archaeal DNA polymerases include without limitation the thermostable and/or thermophilic DNA polymerases such as, for example, DNA polymerases isolated from *Thermus aquaticus* (Taq) DNA polymerase, *Thermus filiformis* (Tfi) DNA polymerase, *Thermococcus zilligi* (Tzi) DNA polymerase, *Thermus thermophilus* (Tth) DNA polymerase, *Thermus flavus* (Tfl) DNA polymerase, *Pyrococcus woesei* (Pwo) DNA polymerase, *Pyrococcus furiosus* (Pfu) DNA polymerase as well as Turbo Pfu DNA polymerase, *Thermococcus litoralis* (Tli) DNA polymerase or Vent DNA polymerase, *Pyrococcus* sp. GB-D polymerase, “Deep Vent” DNA polymerase, New England Biolabs), *Thermotoga maritima* (Tma) DNA polymerase, *Bacillus stearothermophilus* (Bst) DNA polymerase, *Pyrococcus kodakaraensis* (KOD) DNA polymerase, Pfx DNA polymerase, *Thermococcus* sp. JDF-3 (JDF-3) DNA polymerase, *Thermococcus gorgonarius* (Tgo) DNA polymerase, *Thermococcus acidophilium* DNA polymerase; *Sulfolobus acidocaldarius* DNA polymerase; *Thermococcus* sp. 9° N-7 DNA polymerase; *Thermococcus* sp. NA1; *Pyrodictium occultum* DNA polymerase; *Methanococcus voltae* DNA polymerase; *Methanococcus thermoautotrophicum* DNA polymerase; *Methanococcus jannaschii* DNA polymerase; Desulfurococcus strain TOK DNA polymerase (D. Tok Pol); *Pyrococcus abyssi* DNA polymerase; *Pyrococcus horikoshii* DNA polymerase; *Pyrococcus islandicum* DNA polymerase; *Thermococcus fumicolans* DNA polymerase; *Aeropyrum pernix* DNA polymerase; the heterodimeric DNA polymerase DP1/DP2, etc.

[00383] Suitable RNA polymerases include, without limitation, T3, T5, T7, and SP6 RNA polymerases.

[00384] Suitable reverse transcriptases include without limitation reverse transcriptases from HIV, HTLV-I, HTLV-II, FeLV, FIV, SIV, AMV, MMTV and MoMuLV, as well as the commercially available “Superscript” reverse transcriptases, (Life Technologies Corp., Carlsbad, CA) and telomerases.

[00385] In some embodiments, the polymerase is selected from the group consisting of: Phi-29 DNA polymerase, a mutant or variant of Phi-29 DNA polymerase, B103 DNA polymerase and a mutant or variant of B103 DNA polymerase.

[00386] In another aspect, the polymerases can include one or more mutations that improve the performance of the polymerase in the particular biological assay of interest. The mutations can include amino acid substitutions, insertions, or deletions.

[00387] Selecting a Polymerase

[00388] The selection of the polymerase for use in the disclosed methods can be based on the desired polymerase behavior in the particular biological assay of interest. For example, the polymerase can be selected to exhibit enhanced or reduced activity in a particular assay, or enhanced or reduced interaction with one or more particular substrates.

[00389] For example, in some embodiments the polymerase is selected based on the polymerization kinetics of the polymerase either in unconjugated form or when linked to a label (labeled polymerase conjugate). Optionally, the label can be a nanoparticle or fluorescent dye; in some embodiments, the label can be energy transfer donor moiety. For example, the polymerase can be selected on the basis of kinetic behavior relating to nucleotide binding (e.g., association), nucleotide dissociation (intact nucleotide), nucleotide fidelity, nucleotide incorporation (e.g., catalysis), and/or release of the cleavage product. The selected polymerase can be wild-type or mutant.

[00390] In one embodiment, polymerases may be selected that retain the ability to selectively bind complementary nucleotides. In another embodiment, the polymerases may be selected which exhibit a modulated rate (faster or slower) of nucleotide association or dissociation. In another embodiment, the polymerases may be selected which exhibit a reduced rate of nucleotide incorporation activity (e.g., catalysis) and/or a reduced rate of dissociation of the cleavage product and/or a reduced rate of polymerase translocation (after nucleotide incorporation). Some

modified polymerases which exhibit nucleotide binding and a reduced rate of nucleotide incorporation have been described (Rank, U.S. published patent application No. 2008/0108082; Hanzel, U.S. published patent application No. 2007/0196846).

[00391] In polymerases from different classes (including DNA-dependent polymerases), an active-site lysine can interact with the phosphate groups of a nucleoside triphosphate molecule bound to the active site. The lysine residue has been shown to protonate the pyrophosphate leaving-group upon nucleotidyl transfer. Mutant polymerases having this lysine substituted with leucine, arginine, histidine or other amino acids, exhibit greatly reduced nucleotide incorporation rates (Castro, et al., 2009 Nature Structural and Molecular Biology 16:212-218). One skilled in the art can use amino acid alignment and/or comparison of crystal structures of polymerases as a guide to determine which lysine residue to replace with alternative amino acids. The sequences of Phi29 polymerase (SEQ ID NO: 3), RB69 polymerase (SEQ ID NO: 29), an exemplary B103-like polymerase (SEQ ID NO: 33), and Klenow fragment (SEQ ID NO: 3) can be used as the basis for selecting the amino acid residues to be modified (for B103-like polymerase of SEQ ID NO: 33, see, e.g., Hendricks, et al., U.S. Serial No. 61/242,771, filed on September 15, 2009; U.S. Serial No. 61/293,618, filed on January 8, 2010; or Serial No. ____ titled “Polymerase Compositions & Methods”, Attorney Docket No.: LT00052, filed concurrently herewith). In one embodiment, a modified phi29 polymerase can include lysine at position 379 and/or 383 substituted with leucine, arginine or histidine, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3.

[00392] In other embodiments, the polymerase can be selected based on the combination of the polymerase and nucleotides, and the reaction conditions, to be used for the nucleotide binding and/or nucleotide incorporation reactions. For example, certain polymerases in combination with nucleotides which comprise 3, 4, 5, 6, 7, 8, 9, 10 or more phosphate groups can be selected for performing the disclosed methods. In another example, certain polymerases in combination with nucleotides which are linked to an energy transfer moiety can be selected for performing the nucleotide incorporation methods.

[00393] The polymerases, nucleotides, and reaction conditions, can be screened for their suitability for use in the nucleotide binding and/or nucleotide incorporation methods, using well known screening techniques. For example, the suitable polymerase may be capable of binding nucleotides and/or incorporating nucleotides. For example, the reaction kinetics for nucleotide binding, association, incorporation, and/or dissociation rates, can be determined using rapid kinetics techniques (e.g., stopped-flow or quench flow techniques). Using stopped-flow or quench flow techniques, the binding kinetics of a nucleotide can be estimated by calculating the $1/k_d$ value. Stopped-flow techniques which analyze absorption and/or fluorescence spectroscopy properties of the nucleotide binding, incorporation, or dissociation rates to a polymerase are well known in the art (Kumar and Patel 1997 *Biochemistry* 36:13954-13962; Tsai and Johnson 2006 *Biochemistry* 45:9675-9687; Hanzel, U.S. published patent application No. 2007/0196846). Other methods include quench flow (Johnson 1986 *Methods Enzymology* 134:677-705), time-gated fluorescence decay time measurements (Korlach, U.S. Patent No. 7,485,424), plate-based assays (Clark, U.S. published patent application No. 2009/0176233), and X-ray crystal structure analysis (Berman 2007 *EMBO Journal* 26:3494). Nucleotide incorporation by a polymerase can also be analyzed by gel separation of the primer extension products. In one embodiment, stopped-flow techniques can be used to screen and select combinations of nucleotides with polymerases having a t_{pol} value (e.g., $1/k_{pol}$) which is less than a t_{-1} (e.g., $1/k_{-1}$) value. Stopped-flow techniques for measuring t_{pol} (MP Roettger 2008 *Biochemistry* 47:9718-9727; M Bakhtina 2009 *Biochemistry* 48:3197-320) and t_{-1} (M Bakhtina 2009 *Biochemistry* 48:3197-3208) are known in the art.

[00394] For example, some phi29 or B103 polymerases (wild-type or mutant) exhibit t_{pol} values which are less than t_{-1} values, when reacted with nucleotide tetraphosphate or hexaphosphate molecules. In another embodiment, polymerases can be modified by binding it to a chemical compound or an antibody, in order to inhibit nucleotide incorporation.

[00395] In some embodiments, the selection of the polymerase may be determined by the level of processivity desired for conducting nucleotide incorporation or

polymerization reactions. The polymerase processivity can be gauged by the number of nucleotides incorporated for a single binding event between the polymerase and the target molecule base-paired with the polymerization initiation site. For example, the processivity level of the polymerase may be about 1, 5, 10, 20, 25, 50, 100, 250, 500, 750, 1000, 2000, 5000, or 10,000 or more nucleotides incorporated with a single binding event. Processivity levels typically correlate with read lengths of a polymerase. Optionally, the polymerase can be selected to retain the desired level of processivity when conjugated to a label.

[00396] The selection of the polymerase may be determined by the level of fidelity desired, such as the error rate per nucleotide incorporation. The fidelity of a polymerase may be partly determined by the 3' → 5' exonuclease activity associated with a DNA polymerase. The fidelity of a DNA polymerase may be measured using assays well known in the art (Lundburg et al., 1991 *Gene*, 108:1-6). The error rate of the polymerase can be one error per about 100, or about 250, or about 500, or about 1000, or about 1500 incorporated nucleotides. In some embodiments, the polymerase is selected to exhibit high fidelity. Such high-fidelity polymerases include those exhibiting error rates typically of about 5×10^{-6} per base pair or lower.

[00397] In some embodiments, the selection of the polymerase may be determined by the rate of nucleotide incorporation such as about one nucleotide per 2-5 seconds, or about one nucleotide per second, or about 5 nucleotides per second, or about 10 nucleotides per second, or about 20 nucleotides per second, or about 30 nucleotides per second, or more than 40 nucleotides per second, or more than 50-100 per second, or more than 100 per second. In one embodiment, polymerases exhibiting reduced nucleotide incorporation rates include mutant phi29 polymerase having lysine substituted with leucine, arginine, histidine or other amino acids (Castro 2009 *Nature Structural and Molecular Biology* 16:212-218).

[00398] In some embodiments, the polymerase can be selected to exhibit either reduced or enhanced rates of incorporation for polyphosphate-comprising nucleotides comprising a label bonded to the terminal phosphate.

[00399] In some embodiments, the polymerase can be selected to exhibit either reduced or enhanced residence times for a particular nucleotide of interest. In some embodiments, the residence time of the selected polymerase for the particular labeled

nucleotide of interest can be between about 20 msec and about 300 msec, typically between about 55 msec and about 100 msec. In some embodiments, the residence time of the selected polymerase for the particular labeled nucleotide of interest can be between about 1.5 and about 4 times the residence time of the corresponding wild-type polymerase for the labeled nucleotide.

[00400] In some embodiments, the polymerase can be selected, mutated, modified, evolved or otherwise engineered to exhibit either reduced or enhanced entry of nucleotides, particularly labeled nucleotides, into the polymerase active site. Some exemplary polymerases exhibiting altered residence times for labeled nucleotides are disclosed in U.S. Pub. No. 20080108082, published May 8, 2008.

[00401] In some embodiments, the polymerase can be selected to exhibit a reduced K_{sub} for a substrate, particularly a labeled nucleotide analog. In some embodiments, the polymerase can comprise one or more mutations resulting in altered $K_{\text{cat}}/K_{\text{sub}}$ and/or $V_{\text{max}}/K_{\text{sub}}$ for a particular labeled nucleotide. In some embodiments, the $K_{\text{cat}}/K_{\text{sub}}$, the $V_{\text{max}}/K_{\text{sub}}$, or both, are increased as compared to the wild type polymerase.

[00402] Fusion Proteins

[00403] In one aspect, the polymerase can be a fusion protein comprising the amino acid sequence of a nucleic acid-dependent polymerase (the polymerase portion) linked to the amino acid sequence of a second enzyme or a biologically active fragment thereof (the second enzyme portion). The second enzyme portion of the fusion protein may be linked to the amino or carboxyl end of the polymerase portion, or may be inserted within the polymerase portion. The polymerase portion of the fusion protein may be linked to the amino or carboxyl end of the second enzyme portion, or may be inserted within the second enzyme portion. In some embodiments, the polymerase and second enzyme portions can be linked to each other in a manner which does not significantly interfere with polymerase activity of the fusion or with the ability of the fusion to bind nucleotides, or does not significantly interfere with the activity of the second enzyme portion. In the fusion protein, the polymerase portion or the second enzyme portions can be linked with at least one energy transfer donor moiety. The fusion protein can be a recombinant protein having a polymerase

portion and a second enzyme portion. In some embodiments, the fusion protein can include a polymerase portion chemically linked to the second enzyme portion.

[00404] Evolved Polymerases

[00405] The polymerase can be a modified polymerase having certain desired characteristics, such as an evolved polymerase selected from a directed or non-directed molecular evolution procedure. The evolved polymerase can exhibit modulated characteristics or functions, such as changes in: affinity, specificity, or binding rates for substrates (e.g., target molecules, polymerization initiation sites, or nucleotides); binding stability to the substrates (e.g., target molecules, polymerization initiation sites, or nucleotides); nucleotide incorporation rate; nucleotide analog permissiveness; exonuclease activity (e.g., 3'→5' or 5'→3'); rate of extension; processivity; fidelity; stability; or sensitivity and/or requirement for temperature, chemicals (e.g., DTT), salts, metals, pH, or electromagnetic energy (e.g., excitation or emitted energy). Many examples of evolved polymerases having altered functions or activities can be found in U.S. provisional patent application No. 61/020,995, filed January 14, 2008.

[00406] Methods for creating and selecting proteins and enzymes having the desired characteristics are known in the art, and include: oligonucleotide-directed mutagenesis in which a short sequence is replaced with a mutagenized oligonucleotide; error-prone polymerase chain reaction in which low-fidelity polymerization conditions are used to introduce point mutations randomly across a sequence up to about 1 kb in length (R. C. Caldwell, et al., 1992 PCR Methods and Applications 2:28-33; H. Gramm, et al., 1992 Proc. Natl. Acad. Sci. USA 89:3576-3580); and cassette mutagenesis in which a portion of a sequence is replaced with a partially randomized sequence (A. R. Oliphant, et al., 1986 Gene 44:177-183; J. D. Hermes, et al., 1990 Proc. Natl. Acad. Sci. USA 87:696-700; A. Arkin and D. C. Youvan 1992 Proc. Natl. Acad. Sci. USA 89:7811-7815; E. R. Goldman and D. C. Youvan 1992 Bio/Technology 10:1557-1561; Delagrave et al., 1993 Protein Engineering 6: 327-331; Delagrave et al., 1993 Bio/Technology 11: 1548-1555); and domain shuffling.

[00407] Methods for creating evolved antibody and antibody-like polypeptides can be adapted for creating evolved polymerases, and include applied molecular evolution

formats in which an evolutionary design algorithm is applied to achieve specific mutant characteristics. Many library formats can be used for evolving polymerases including: phage libraries (J. K. Scott and G. P. Smith 1990 *Science* 249:386-390; S. E. Cwirla, et al. 1990 *Proc. Natl. Acad. Sci. USA* 87:6378-6382; J. McCafferty, et al. 1990 *Nature* 348:552-554) and *lacI* (M. G. Cull, et al., 1992 *Proc. Natl. Acad. Sci. USA* 89:1865-1869).

[00408] Another adaptable method for evolving polymerases employs recombination (crossing-over) to create the mutagenized polypeptides, such as recombination between two different plasmid libraries (Caren et al. 1994 *Bio/Technology* 12: 517-520), or homologous recombination to create a hybrid gene sequence (Calogero, et al., 1992 *FEMS Microbiology Lett.* 97: 41-44; Galizzi et al., WO91/01087). Another recombination method utilizes host cells with defective mismatch repair enzymes (Radman et al., WO90/07576). Other methods for evolving polymerases include random fragmentation, shuffling, and re-assembly to create mutagenized polypeptides (published application No. U.S. 2008/0261833, Stemmer). Adapting these mutagenesis procedures to generate evolved polymerases is well within the skill of the art.

[00409] In some embodiments, the polymerase can be fused with, or otherwise engineered to include, DNA-binding or other domains from other proteins that are capable of modulating DNA polymerase activity. For example, fusion of suitable portions of the Single-Stranded DNA Binding Protein (SSBP), thioredoxin and/or T7 DNA polymerase to bacterial or viral DNA polymerases has been shown to enhance both the processivity and fidelity of the DNA polymerase. Similarly, other groups have described efforts to engineer polymerases so as to broaden their substrate range. See, e.g., Ghadessy et al, *Nat. Biotech.*, 22 (6):755-759 (2004). Similarly, the conjugates of the present disclosure can optionally comprise any polymerase engineered to provide suitable performance characteristics, including for example a polymerase fused to intact SSBP or fragments thereof, or to domains from other DNA-binding proteins (such as the herpes simplex virus UL42 protein.)

[00410] In some embodiments, a blend of different conjugates, each of which comprises a polymerase of unique sequence and characteristics, can be used according to the methods described herein. Use of such conjugate blends can

additionally increase the fidelity and processivity of DNA synthesis. For example, use of a blend of processive and non-processive polymerases has been shown to result in increased overall read length during DNA synthesis, as described in U.S. Published App. No. 2004/0197800. Alternatively, conjugates comprising polymerases of different affinities for specific acceptor-labeled nucleotides can be used so as to achieve efficient incorporation of all four nucleotides.

[00411] In one embodiment, the polymerase can be a mutant which retains nucleotide polymerization activity but lacks the 3'→5' or 5' → 3' exonuclease activity. In another embodiment, the polymerase can be an exonuclease minus mutant which is based on wild type phi29 polymerase of SEQ ID NO: 1 (Blanco, U.S. Patent No. 5,001,050, 5,198,543, and 5,576,204; and Hardin PCT/US2009/31027 with an International filing date of January 14, 2009) and comprising one or more substitution mutations, including: D12A, D66A, D169A, H61R, N62D, Q380A, and/or S388G, and any combination thereof.

[00412] In some embodiments, the polymerase can comprise the amino acid sequence of any polymerase disclosed in U.S. Provisional Application Nos. 61/242,771, filed on September 15, 2009; 61/263,974, filed on November 24, 2009 and 61/299,919, filed on January 29, 2010, or any variant thereof.

[00413] In some embodiments, the polymerase is an *E. coli* K12 DNA polymerase I having the following amino acid sequence:

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1  MVQIPQNPLI  LVDGSSYLIR  AYHAFPLTN  SAGEPTGAMY  GVLNMLRSLI  MQYKPTHAAV
61  VFDAKGKTFR  DELFEHYKSH  RPPMPDDLRA  QIEPLHAMVK  AMGLPLLAVS  GVEADDVIGT
121 LAREAEKAGR  PVLISTGDKD  MAQLVTPNIT  LINTMTNTIL  GPEEVVNKYG  VPELIIDFL
181 ALMGDSSDNI  PGVPGVGEXT  AQALLQGLGG  LDTLYAEPEK  IAGLSFRGAK  TMAAKLEQNK
241 EVAYLSYQLA  TIKTDVELEL  TCEQLEVQQP  AAEELLGLFK  KYEFKRWTAD  VEAGKWLQAK
301 GAKPAAKPQE  TSVADEAPEV  TATVISYDNY  VTILDEETLK  AWIAKLEKAP  VFAFDTETDS
361 LDNISANLVG  LSFAIEPGVA  AYIPVAHDYL  DAPDQISRER  ALELLKPLLE  DEKALKVGQN
421 LKYDRGILAN  YGIELRGIAF  DTMLESYILN  SVAGRHDMD  LAERWLKHK  ITFEEIAGKG
481 KNQLTFNQIA  LEEAGRYAAE  DADVTLQLHL  KMWPDQLQHK  GPLNVFENIE  MPLVPVLSRI
541 ERNGVKIDPK  VLHNHSEELT  LRLAELEKKA  HEIAGEEFNL  SSTKQLQTIL  FEKQGIKPLK
601 KTPGGAPSTS  EEVLEELALD  YPLPKVILEY  RGLAKLKSTY  TDKLPLMINP  KTGRVHTSYH
661 QAVTATGRLS  STDPNLQNIP  VRNEEGRIR  QAFIAPEDYV  IVSADYSQIE  LRIMAHLSRD
721 KGLLTAFAGE  KDIHRATAAE  VFGLPLET  SEQRRSAKAI  NFGLIYGMSA  FGLARQLNIP
781 RKEAQKYMDL  YFERYPGVLE  YMERTRAQAK  EQGYVETLDG  RRLYLPIK  SNGARRAAAE

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841 RAAINAPMQG TAADIIKRAM IAVDAWLQAE QPRVRMIMQV HDELVFEVHK DDVDAVAKQI
 901 HQLMENCTRL DVPLLVEVGS GENWDQAH

(SEQ ID NO: 1)

[00414] In some embodiments, the polymerase can comprise an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or a biologically active fragment thereof.

[00415] In some embodiments, the polymerase comprises an amino acid sequence at least 70% identical to the amino acid sequence of DNA polymerase I (SEQ ID NO: 1) or Klenow DNA polymerase (SEQ ID NO: 2), wherein the cysteine residue corresponding to the cysteine residue at position 907 is mutated to a serine or some other residue, the numbering being relative to *E. coli* K12 DNA polymerase I (SEQ ID NO: 1). In some embodiments, the mutant, variant, mutated or otherwise mutated polymerase lacks 3' to 5' exonuclease activity.

[00416] In some embodiments, the polymerase comprises an amino acid sequence at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid of DNA polymerase I (SEQ ID NO: 1) or Klenow DNA polymerase (SEQ ID NO: 2) and further comprises one or more substitutions wherein any amino acid residue is substituted with an engineered cysteine residue, which can serve as an attachment site for a label. Optionally, the label can be attached to the engineered cysteine residue using the linking agent SMCC.

[00417] In some embodiments, the engineered cysteine residue is substituted for the threonine residue at position 748, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1)

[00418] In some embodiments, the engineered cysteine residue is substituted for the threonine residue at position 750, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00419] In some embodiments, the engineered cysteine residue is substituted for the serine residue at position 751, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00420] In some embodiments, the engineered cysteine residue is substituted for the asparagine residue at position 778, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00421] In some embodiments, the engineered cysteine residue is substituted for the glycine residue at position 730, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00422] In some embodiments, the engineered cysteine residue is substituted for the asparagine residue at position 922, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00423] In some embodiments, the engineered cysteine residue is substituted for the Q (glutamine) residue at position 926, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00424] In some embodiments, the engineered cysteine residue is substituted for the alanine residue at position 927, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00425] In some embodiments, the engineered cysteine residue is substituted for the histidine residue at position 928, the numbering being relative to wild-type *E. coli* K12 DNA polymerase (SEQ ID NO: 1).

[00426] In some embodiments, the polymerase can comprise the Klenow form of DNA polymerase. In some embodiments, the polymerase comprises the Klenow truncated form of *E. coli* K12 DNA polymerase I (“Klenow DNA polymerase”) having the following sequence:

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MVISYDNY VTILDEETLK AWIAKLEKAP VFAPDTETDS
361 LDNISANLVG LSFAIEPGVA AYIPVAHDYL DAPDQISRER ALELLKPLLE DEKALKVGQN
421 LKYDRGILAN YGIELRGIAF DTMLESYILN SVAGRHDMDL LAERWLKHKI ITFEEIAGKG
481 KNQLTFNQIA LEEAGRYAAE DADVTQLQHL KMWPDQLQHK GPLNVFENIE MPLVPVLSRI
541 ERNGVKIDPK VLHNHSEELT LRLAELEKKA HEIAGEEFNL SSTKQLQTIIL FEKQGIKPLK
601 KTPGGAPSTS EEVLEELALD YPLPKVILEY RGLAKLKSTY TDKLPLMINP KTGRVHTSYH
661 QAVTATGRLS STDPNLQNIP VRNEEGRRIR QAFIAPEDYV IVSADYSQIE LRIMAHLSRD
721 KGLLTAFAGE KDIHRATAAE VFGLPLETVT SEQRRSAKAI NFGLIYGMSA FGLARQLNIP
781 RKEAQKYMDL YFERYPGVLE YMERTRAQAK EQGYVETLDG RRLYLPIKIS SNGARRAAAE
841 RAAINAPMQG TAADI IKRAM IAVDAWLQAE QPRVRMIMQV HDELVFVHK DDVDAVAKQI
901 HQLMENCTRL DVPLLVEVGS GENWDQAH

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(SEQ ID NO: 2)

[00427] In some embodiments, the polymerase can comprise an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or a biologically active fragment thereof.

[00428] In some embodiments, the polymerase of the conjugate is a mutant or variant Klenow form of DNA polymerase comprising amino acid sequence at least about 85% identical to the amino acid sequence of SEQ ID NO: 2, or a biologically active fragment thereof. Optionally, the polymerase lacks 3' to 5' exonuclease activity.

[00429] In some embodiments, the modified polymerase is derived from a polymerase of any member of the Phi-29-like family of phages. The Phi-29-like phages are a genus of phages that are related to Phi-29 that includes the phages PZA, Φ15, BS32, B103, M2Y (M2), Nf1 and GA-1. Phages of this group have been sub-classified into three groups based on serological properties, DNA and/or polymerase maps and partial or complete DNA sequences, and share several characteristics in common. For example, such phages can typically undergo protein-primed DNA replication. See, for example, Meijer et al., "Phi-29 family of phages" Microbiol. & Mol. Biol. Revs. 65(2):261-287 (2001).

[00430] In some embodiments, the polymerase is homologous to a polymerase of one or more of the following organisms: B103, Phi-29, GA-1, PZA, Phi-15, BS32, M2Y, Nf, G1, Cp-1, PRD1, PZE, SF5, Cp-5, Cp-7, PR4, PR5, PR722, or L17. See, e.g., Meijer et al., "Phi-29 family of phages," Microbiol. & Mol. Biol. Revs. 65(2):261-287 (2001).

[00431] In some embodiments, the polymerase can comprise the Phi-29 DNA polymerase or a biologically active fragment thereof. (See, e.g., U.S. Patent Nos. 5,001,050; 5,198,543 and 5,576,204). Typically, the Phi-29 polymerase comprises the following sequence:

MKHMPRKMY	CDFETTTKVE	DCRVWAYGYM	NIEDHSEYKI	GNSLDEFMAW	VLKVQADLYF
70	80	90	100	110	120
HNLKFDGAFI	INWLERNGFK	WSADGLPNTY	NTIISRMGQW	YMIDICLGKY	GKRKIHTVIY
130	140	150	160	170	180
DSLKKLPFPV	KKIAKDFKLT	VLKGDIDYHK	ERPVGYKITP	EEYAYIKNDI	QIIAEALLIQ
190	200	210	220	230	240
FKQGLDRMTA	GSDSLKGFKD	IITTKFKKKV	FPTLSLGLDK	EVRYAYRGGF	TWLNDRFKEK
250	260	270	280	290	300
EIGEGMVFDV	NSLYPAQMYS	RLLPYGEPIV	FEGKYVWDED	YPLHIQHIRC	EFELKEGYIP
310	320	330	340	350	360
TIQIKRSRFY	KGNEYLKSSG	GEIADLWLSN	VDLELMKEHY	DLYNVEYISG	LKFKATTGLF

370	380	390	400	410	420
KDFIDKWTYI	KITSEGAIKQ	LAKLMLNSLY	GKFASNPDVT	GKVPYLKENG	ALGFRLGEEE
430	440	450	460	470	480
TKDPVYTPMG	VFITAWARYT	TITAAQACYD	RIIYCDTDSI	HLTGTEIPDV	IKDIVDPKKL
490	500	510	520	530	540
GYWAHESTFK	RAKYLRQKTY	IQDIYMKEVD	GKLVEGSPDD	YTDIKFSVKC	AGMTDKIKKE
550	560	570			
VTFENFKVGF	SRKMKPKPVQ	VPGGVVLVDD	TFTIK		

(SEQ ID NO: 3)

[00432] In some embodiments, the polymerase can comprise an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3.

[00433] In some embodiments, the polymerase is derived from a Phi-29-like polymerase and comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 and further including one or more amino acid mutations at positions selected from the group consisting of: 132, 135, 250, 266, 332, 342, 368, 370, 371, 372, 373, 375, 379, 380, 383, 387, 390, 458, 478, 480, 484, 486 and 512, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the Phi-29-like polymerase can comprise an amino acid deletion, wherein the deletion includes some of all of the amino acids spanning positions 306 to 311.

[00434] Without being bound to any particular theory, it is thought that the domain comprising amino acid residues 304-314 of the amino acid sequence of SEQ ID NO: 3 (Phi-29 polymerase), or homologs thereof, can reduce or otherwise interfere with DNA initiation and/or elongation by inhibiting access to the Phi-29 polymerase active site, and that this region must be displaced in order to allow access to the active site. See, e.g., Kamtekar et al., "The Φ29 DNA polymerase:protein primer structure suggests a model for the initiation to elongation transition", EMBO J., 25:1335-1343 (2005).

[00435] In some embodiments, the polymerase is derived from a Phi-29-like polymerase and comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 and further includes one or more amino acid mutations selected from the group

consisting of: K132A, K135A, K135D, K135E, V250A, V250C, Y266F, D332Y, L342G, T368D, T368E, T368F, K370A, K371E, T372D, T372E, T372R, T372K, T373A, T373F, T373H, T373K, T373Q, T373R, T373S, T373W, T373Y, T373A, T373E, E375A, E375F, E375H, E375K, E375Q, E375R, E375S, E375W, E375Y, K379A, Q380A, K383E, K383H, K383L, K383R, N387Y, Y390F, D458N, K478D, K478E, K478R, L480K, L480R, A484E, E486A, E486D, K512A, K512D, K512E, K512R, K512Y, K371E/K383E/N387Y/D458N, Y266F/Y390F, Y266F/Y390F/K379A/Q380A, K379A/Q380A, E375Y/Q380A/K383R, E375Y/Q380A/K383H, E375Y/Q380A/K383L, E375Y/Q380A/V250A, E375Y/Q380A/V250C, E375Y/K512Y/T368F, E375Y/K512Y/T368F/A484E, K379A/E375Y, K379A/K383R, K379A/K383H, K379A/K383L, K379A/Q380A, V250A/K379A, V250A/K379A/Q380A, V250C/K379A/Q380A, K132A/K379A and deletion of some or all of the amino acid residues spanning R306 to K311, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the mutant Phi-29-like polymerase can exhibit increased branching ratio and/or increased t_{-1} value in the presence of dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 3. In some embodiments, the branching ratio and/or t_{-1} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the branching ratio and/or t_{-1} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D12A, E14I, E14A, T15I, N62D, D66A, Y165F, Y165C, and D169A, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3.

[00436] In some embodiments, the polymerase is derived from a Phi-29-like polymerase and comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3.

[00437] In other embodiments, the polymerase of the conjugate is a mutant or variant Phi-29 DNA polymerase comprising an N-terminal polyhistidine tag (His-tag) fused

to an amino acid sequence at least about 85% identical to a Phi-29 DNA polymerase comprising the amino acid sequence of SEQ ID NO: 3, or biologically active fragment thereof. Optionally, the polymerase lacks 3' to 5' exonuclease activity. In some embodiments, the enzyme is a Klenow DNA polymerase having the amino acid sequence of SEQ ID NO: 2 and further comprising an engineered cysteine introduced at amino acid positions 730, 748, 750, 751, 778, 922, 926, 927 and 928, or any combination thereof. In some embodiments, the enzyme is Phi-29 DNA polymerase having the amino acid sequence of SEQ ID NO: 3, and the cysteine at amino acid position 473 serves as an attachment site for the label.

[00438] In some embodiments, the polymerase can be a deletion mutant which retains nucleotide polymerization activity but lacks the 3' → 5' or 5' → 3' exonuclease activity. For example, mutant phi29 polymerases having exonuclease-minus activity, or reduced exonuclease activity, can optionally comprise the amino acid sequence of SEQ ID NO: 3 and further comprise one or more amino acid substitutions at positions selected from the group consisting of: 12, 14, 15, 62, 66, 165 and 169 (wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3).

[00439] In some embodiments, the polymerase is derived from a Phi-29-like polymerase and comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 3 and comprises one or more of the following amino acid substitutions: D12A, E14I, E14A, T15I, N62D, D66A, Y165F, Y165C, and D169A, wherein the numbering is relative to SEQ ID NO: 3.

[00440] In some embodiments, the conjugate can comprise at least one biomolecule linked to a label through a peptide linker comprising a series of amino acid residues.

[00441] In some embodiments, the peptide linker can comprise the amino acid sequence: LLGAAAKGAAAKGSAA (SEQ ID NO: 4)

[00442] This linker is hereinafter referred to as the "H-linker".

[00443] In some embodiments, the peptide linker can comprise the amino acid sequence: LLGGGGSGGGGSAAAGSAA (SEQ ID NO: 5)

[00444] This linker is hereinafter referred to as the "F-linker".

[00445] Optionally, the peptide linker can be fused to the N-terminus of the biomolecule, the C-terminus of the biomolecule, or any suitable position along the length of the biomolecule.

[00446] In some embodiments, the conjugate comprises a protein or biologically active fragment thereof linked to a label, wherein the protein comprises one or more cysteine replacements, i.e., one or more cysteine residues of the protein have been selectively replaced through mutation, deletion or other suitable modification so as to reduce the number of thiol residues capable of acting as points of covalent attachment for the label. For example, the polymerase can comprise the Klenow form of DNA polymerase, having the sequence of SEQ ID NO: 2, or biologically active fragment or variant thereof. Klenow DNA polymerase typically comprises a single cysteine residue at amino acid position 907 of the protein. In some embodiments, this residue is selectively mutated to another residue, for example, serine. Alternatively, the polymerase can comprise Phi-29 DNA polymerase or variant thereof, having the sequence of SEQ ID NO: 3, which typically comprises at least seven different cysteine residues. In some embodiments, some or all of these cysteine residues are selectively mutated so as to replace them with another residue. For example, the polymerase can be a protein comprising an amino acid sequence that is 60%, 70%, 80%, 85%, 90%, 95% or 100% identical to the amino acid sequence of SEQ ID NO: 3, wherein cysteine residues at amino acid positions 47, 315, and 555 have been selectively replaced with non-cysteine residues.

[00447] In some embodiments, the protein can optionally be fused with a polycysteine tag comprising multiple cysteine residues, and the cysteine residues of the poly-cysteine tag can serve as sites of attachment of the label mediated by SMCC. For example, the biomolecule can be a fusion protein that comprises a polycysteine tag fused to the open reading frame of a protein or biologically active fragment thereof. The polycysteine tag can comprise a stretch of 6, 7, 8, 9, 10, 11, 12 or more consecutive cysteine residues. The polycysteine tag can be fused to the N-terminus, the C-terminus or any other suitable position of the protein.

[00448] Optionally, a polycysteine tag can be separated from the amino acid residues of the protein by a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 4 and/or SEQ ID NO: 5.

[00449] In some embodiments, the fusion protein comprises a polycysteine tag fused to the N-terminus of the Klenow form of E. coli DNA polymerase. In some embodiments, the polycysteine tag and the Klenow polymerase peptide can be separated by a peptide linker. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MCCCCCCCC	CCCLLGGGGS	GGGGSAAAGS	AARKMYSCDF	ETTTKVEDCR	VWAYGYMNIE
70	80	90	100	110	120
DHSEYKIGNS	LDEFMAWVLK	VQADLYFHNL	KFDGAFIINW	LERNGFKWSA	DGLPNTYNTI
130	140	150	160	170	180
ISRMGQWYMI	DICLGYGKGR	KIHTVIYDSL	KKLPFPVKKI	AKDFKLTVLK	GDIDYHKERP
190	200	210	220	230	240
VGKITPEEY	AYIKNDIQII	AEALLIQFKQ	GLDRMTAGSD	SLKGFKDIIT	TKKFKKVFP
250	260	270	280	290	300
LSLGLDKEVR	YAYRGGFTWL	NDRFKEKEIG	EGMVFDVNSL	YPAQMYSRLL	PYGEPIVFEG
310	320	330	340	350	360
KYVWDEDYPL	HIQHIRCEFE	LKEGYIPTIQ	IKRSRFYKGN	EYLKSSGGEI	ADLWLSNVDL
370	380	390	400	410	420
ELMKEHYDLY	NVEYISGLKF	KATTGLFKDF	IDKWTYIKTT	SEGAIKQLAK	LMLNSLYGKF
430	440	450	460	470	480
ASNPDVTKGV	PYLKENGALG	FRLGEEETKD	PVYTPMGVFI	TAWARYTTIT	AAQACYDRII
490	500	510	520	530	540
YCDTDSIHLT	GTEIPDVIKD	IVDPKKLGyw	AHESTFKRAK	YLRQKTYIQD	IYMKEVDGKL
550	560	570	580	590	600
VEGSPDDYTD	IKFSVKCAGM	TDKIKKEVTF	ENFKVGFSRK	MKPKPVQVPG	GVVLVDDTFT

IK

(SEQ ID NO: 6)

[00450] In some embodiments, covalent conjugation of a protein to a label comprising one or more carboxyl groups on its surface can be achieved through use of the homobifunctional cross-linking agent Bis(sulfosuccinimidyl)suberate (BS3), which can be useful in linking amines to amines. BS3 contains an amine-reactive N-hydroxysulfosuccinimide (NHS) ester at each end of an 8-carbon spacer arm. NHS esters can react with primary amines at pH 7-9 to form stable amide bonds, along with release of the N-hydroxysulfosuccinimide leaving group. Various proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for NHS-ester crosslinking reagents. Alternatively, the protein can be conjugated to a poly-lysine tag comprising multiple lysine residues, and the lysine residues of the poly-

lysine tag can serve as sites of attachment of the label mediated by BS3. In some embodiments, the biomolecule is a fusion protein and comprises a polylysine tag fused to the open reading frame of a protein or biologically active fragment thereof. The polylysine tag can comprise a stretch of 6, 7, 8, 9, 10, 11, 12 or more consecutive lysine residues. The polylysine tag can be fused to the N-terminus, the C-terminus or any other suitable position of the protein. Optionally, a polylysine tag can be separated from the amino acid residues of the protein by a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 5.

[00451] In some embodiments, the polymerase of the conjugate is fused with a polylysine tag at its N-terminus, and then linked to labels coated with amine groups (e.g., PEG-amine) using the linking agent Bis(sulfosuccinimidyl)suberate (BS3), which is useful in linking amines to amines.

[00452] In some embodiments, the fusion protein comprises a polylysine tag fused to the N-terminus of the Klenow form of E. coli DNA polymerase. In some embodiments, the polylysine tag and the Klenow polymerase peptide are separated by a peptide linker. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MKKKKKKKKK	KKKLLGGGGS	GGGGSAAAGS	AARKMYSCDF	ETTTKVEDCR	VWAYGYMNIE
70	80	90	100	110	120
DHSEYKIGNS	LDEFMAWVLK	VQADLYFHNL	KFDGAFIINW	LERNGFKWSA	DGLPNTYNTI
130	140	150	160	170	180
ISRMGQWYMI	DICLGYGKGR	KIHTVIYDSL	KKLPFPVKKI	AKDFKLTVLK	GDIDYHKERP
190	200	210	220	230	240
VGKITPEEY	AYIKNDIQII	AEALLIQFKQ	GLDRMTAGSD	SLKGFKDIIT	TKKFKKVFP
250	260	270	280	290	300
LSLGLDKEVR	YAYRGGFTWL	NDRFKEKEIG	EGMVFDVNSL	YPAQMYSRLL	PYGEPIVFEG
310	320	330	340	350	360
KYVWDEDYPL	HIQHIRCEFE	LKEGYIPTIQ	IKRSRFYKGN	EYLKSSGGEI	ADLWLSNVDL
370	380	390	400	410	420
ELMKEHYDLY	NVEYISGLKF	KATTGLFKDF	IDKWTYIKTT	SEGAIKQLAK	LMLNSLYGKF
430	440	450	460	470	480
ASNPDVTGKV	PYLKENGALG	FRLGEEETKD	PVYTPMGVFI	TAWARYTTIT	AAQACYDRII
490	500	510	520	530	540
YCDTDSIHLT	GTEIPDVIKD	IVDPKKLGYW	AHESTFKRAK	YLRQKTYIQD	IYMKEVDGKL
550	560	570	580	590	600
VEGSPDDYTD	IKFSVKCAGM	TDKIKKEVTF	ENFKVGFSRK	MKPKPVQVPG	GVVLVDDTFT

IK

(SEQ ID NO: 7)

[00453] In some embodiments, the biomolecule is a protein that is fused or otherwise coupled to a Transglutaminase tag comprising the amino acid sequence PKPQQF, which can be used as a site of attachment for amine reactive groups mediated by the enzyme transglutaminase. The transglutaminase tag can be fused to the N-terminus, the C-terminus or any other suitable position of the protein. Optionally, a transglutaminase tag can be separated from the amino acid residues of the protein by a peptide linker.

[00454] In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

MHHHHHLLG	GGGSGGGGSA	AAPKPQQFGS	AARKMYSCDF	ETTTKVEDCR	VWAYGYMNIE
70	80	90	100	110	120
DHSEYKIGNS	LDEFMAWVLK	VQADLYFHNL	KFDGAFIINW	LERNGFKWSA	DGLPNTYNTI
130	140	150	160	170	180
ISRMGQWYMI	DICLGYGKGR	KIHTVIYDSL	KKLPFPVKKI	AKDFKLTVLK	GDIDYHKERP
190	200	210	220	230	240
VGKITPEEY	AYIKNDIQII	AEALLIQFKQ	GLDRMTAGSD	SLKGFKDIIT	TKKFKKVFPPT
250	260	270	280	290	300
LSLGLDKEVR	YAYRGGFTWL	NDRFKEKEIG	EGMVFDVNSL	YPAQMYSRLL	PYGEPVFEFEG
310	320	330	340	350	360
KYVWDEDYPL	HIQHIRCEFE	LKEGYIPTIQ	IKRSRFYKGN	EYLKSSGGEI	ADLWLSNVDL
370	380	390	400	410	420
ELMKEHYDLY	NVEYISGLKF	KATTGLFKDF	IDKWTYIKTT	SEGAIKQLAK	LMLNSLYGKF
430	440	450	460	470	480
ASNPDVTGKV	PYLKENGALG	FRLGEEETKD	PVYTPMGVFI	TAWARYTTIT	AAQACYDRII
490	500	510	520	530	540
YCDTDSIHLT	GTEIPDVIKD	IVDPKKLGYW	AHESTFKRAK	YLRQKTYIQD	IYMKEVDGKL
550	560	570	580	590	600
VEGSPDDYTD	IKFSVKCAGM	TDKIKKEVTF	ENFKVGFSRK	MKPKPVQVPG	GVVLVDDTFT

IK

(SEQ ID NO: 8)

[00455] In some embodiments, covalent conjugation of a biomolecule to a label can be accomplished via use of a Protein Kinase A (PKA) site fused, inserted or otherwise engineered into the biomolecular structure, which can permit more selectivity in choosing a point of attachment for the label to the biomolecule. For example, although a given protein may have several primary amines and cysteine thiols available for covalent conjugation, attempted modification of these reactive groups is not specific for one particular primary amine or thiol and frequently modification of the primary amine or thiol can result in decreased activity of the protein. Another

method of conjugation that avoids such problems involves the engineering of a Protein Kinase A recognition sequence, typically comprising the amino acid sequence LRRASLG, into the protein at a desired location. After incubation of the engineered protein with Protein Kinase A and ATP- γ S, the protein will contain a single reactive phosphorothioate at the desired location. This single phosphorothioate can be selectively modified to create a covalent conjugate linked at the sulfur atom of the phosphorothioate.

[00456] In some embodiments, the protein containing the single phosphorothioate can be covalently conjugated to labels containing residual carboxylate groups on their surface using the following synthetic route: The labels are first modified with an excess of adipic dihydrazide via EDC coupling. After purification, the hydrazide functionalized labels are then reacted in the dark with an excess of iodoacetic acid also using EDC as the coupling agent. The resulting purified product comprises an iodoacetal functional group that is reactive with thiols and phosphorothioates. Consequently, in the final reaction an excess of the phosphorothioate-containing protein is incubated with iodoacetal modified labels at pH 5.5. The reaction product can be purified by size exclusion chromatography and characterized for activity and binding.

[00457] In some embodiments, the biomolecule is a protein that is fused or otherwise coupled to a protein kinase A (PKA) tag comprising the amino acid sequence LRRASL, which can be used as a site of attachment mediated by the enzyme protein kinase A enzyme.

[00458] The PKA tag can be fused to the N-terminus, the C-terminus or any other suitable position of the protein. Optionally, the PKA tag can be separated from the amino acid residues of the protein by a linker, typically a peptide linker. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MGLRRASLHH	LLGGGGSGGG	GSAAAGSAAR	KMYSCDFETT	TKVEDCRVWA	YGYMNIEDHS
70	80	90	100	110	120
EYKIGNSLDE	FMAWVLKVQA	DLYFHNLFKD	GAFIINWLER	NGFKWSADGL	PNTYNTIISR
130	140	150	160	170	180
MGQWYMIDIC	LGYGKGRKIH	TVIYDSLKKL	PFPPVKKIAD	FKLTVLKGDI	DYHKERPVGY

190	200	210	220	230	240
KITPEEYAYI	KNDIQIIAEA	LLIQFKQGLD	RMTAGSDSLK	GFKDIIITKK	FKKVFPITLSL
250	260	270	280	290	300
GLDKEVRYAY	RGGFTWLNDR	FKEKEIGEGM	VFDVNSLYPA	QMYSRLLPYG	EPIVFEGKYV
310	320	330	340	350	360
WDEDYPLHIQ	HIRCEFELKE	GYIPTIQIKR	SRFYKGNEYL	KSSGGEIADL	WLSNVDLELM
370	380	390	400	410	420
KEHYDLYNVE	YISGLKFKAT	TGLFKDFIDK	WTYIKTTSEG	AIKQLAKLML	NSLYGKFASN
430	440	450	460	470	480
PDVTGKVPYL	KENGALGFRL	GEEETKDPVY	TPMGVFITAW	ARYTTITAAQ	ACYDRIIYCD
490	500	510	520	530	540
TDSIHLTGTE	IPDVIKDIDV	PKKLGywaHE	STFKRAKYLR	QKTYIQDIYM	KEVDGKLVEG
550	560	570	580	590	
SPDDYTDIKF	SVKCAGMTDK	IKKEVTFENF	KVGFSRKMKP	KPVQVPGGVV	LVDDTFITIK

(SEQ ID NO: 9)

[00459] In some embodiments, the biomolecule comprises a Phi-29 polymerase further comprise a biotin ligase recognition sequence and optionally including a His-tag. The biotin ligase site and/or optionally the His-tag can be located at the N-terminus, the C-terminus or any other suitable position of the Phi-29 polymerase. Optionally, the biotin ligase sequence and/or the His-tag can be separated from the amino acid residues of the Phi-29 protein by a linker, typically a peptide linker. In some embodiments, the biotin ligase recognition site comprises a biotin acceptor peptide. In some embodiments, the biotin acceptor site can comprise the amino acid sequence of SEQ ID NO: 10:

[00460] GLNDIFEAQKIEWHE (SEQ ID NO: 10)

[00461] See, e.g., Howarth et al., "Targeting quantum dots to surface proteins in living cells with biotin ligase", Proc. Natl. Acad. Sci. USA 102(21):7583-7588 (2005).

[00462] In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

MSHHHHHSMGLNDIFEAQKIEWHEGAPGARGSKHMPRKMYSCAFETTTKVEDCRVWAYGYMNIEDHS
EYKIGNSLDEFMAWVLKVQADLYFHNLFAGAFIINWLERNGFKWSADGLPNTYNTIISRMGQWYMIDI
CLGYKGKRKIHTVIYDSLKKLPFPVKKIADFKLTVLKGDDIDYHKERPVGyKITPEEYAYIKNDIQIIA
EALLIQFKQGLDRMTAGSDSLKGFKDIIITKKFKKVFPITLSLGLDKEVRYAYRGGFTWLNDRFKEKEIG
EGMVFDVNSLYPAQMYSRLLPYGEPIVFEGKYVWDEDYPLHIQHIRCEFELKEGYIPTIQIKRSRFYKG
NEYLKSSGGEIADLWLSNVDLELMKEHYDLYNVEYISGLKFKATTGLFKDFIDKWTYIKTTSEGAIKQL

AKLMLNSLYGKFASNP DVTGKVPY LKENGALG FRLGEEETKDPVYTPMGVFITAWARYTTITAAQACYD
 RIIYCDTDSIHLTGTEIPDVIKDIVDPKKLG YWAHESTFKRAKYLRQKTYIQDIYMKEVDGKLVEGSPD
 DYTDIKFSVKCAGMTDKIKKEVTFENFKVGF SRKMKPKPVQVPGGVVLVDDTFTIK

(SEQ ID NO: 11)

[00463] Hereinafter, the protein of SEQ ID NO: 11 is referred to variously as “HBP1” or HBP-1.” It comprises a His-tagged Phi-29 polymerase peptide comprising a biotin ligase site that is fused to the N-terminus of the Phi-29 polymerase, which is exonuclease-minus and includes the D12A and D66A mutations. In some embodiments, the polymerase of the labeled polymerase conjugate comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 11.

[00464] In some embodiments, the polymerase comprises a fusion protein having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence, which comprises a Phi-29 polymerase peptide comprising an N-terminal His-tag and an intervening H-linker sequence, as follows:

MHHHHHLLG	AAAKGAAAG	SAARKMYSCD	FETTTKVEDC	RVWAYGYMNI	EDHSEYKIGN
70	80	90	100	110	120
SLDEFMAWVL	KVQADLYFHN	LKFDGAFIIN	WLERNGFKWS	ADGLPNTYNT	IISRMGQWYM
130	140	150	160	170	180
IDICLGKYGK	RKIHTVIYDS	LKKLPFPVKK	IAKDFKLTIVL	KGDIDYHKER	PVGYKITPEE
190	200	210	220	230	240
YAYIKNAIQI	IAEALLIQFK	QGLDRMTAGS	DSLKGFKDII	TTKKFKKVFP	TLSLGLDKEV
250	260	270	280	290	300
RYAYRGGFTW	LNDRFKEKEI	GEGMVFDVNS	LYPAQMYSRL	LPYGEPVFE	GKYVWDEDYP
310	320	330	340	350	360
LHIQHIRCEF	ELKEGYIPTI	QIKRSRFYKG	NEYLKSSGGE	IADLWLSNVD	LELMKEHYDL
370	380	390	400	410	420
YNVEYISGLK	FKATTGLFKD	FIDKWTYIKT	TSEGAIKQLA	KLMLNSLYGK	FASNP DVTGK
430	440	450	460	470	480
VPY LKENGAL	GFRLGEEETK	DPVYTPMGVF	ITAWARYTTI	TAAQACYDRI	IYCDTDSIHL
490	500	510	520	530	540
TGTEIPDVIK	DIVDPKKLG Y	WAHESTFKRA	KYLRQKTYIQ	DIYMKEVDGK	LVEGSPDDYT
550	560	570	580	590	
DIKFSVKCAG	MTDKIKKEVT	FENFKVGF SR	KMKPKPVQVP	GGVVLVDDTF	TIK

(SEQ ID NO: 12)

[00465] In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following

amino acid sequence, which comprises a Phi-29 polymerase peptide comprising an N-terminal His-tag and an intervening F-linker sequence, as follows:

MHHHHHLLG	GGGSGGGGSA	AAGSAARKMY	SCDFETTTKV	EDCRVWAYGY	MNIEDHSEYK
70	80	90	100	110	120
IGNSLDEFMA	WVLKVQADLY	FHNLKFDGAF	IINWLERNGF	KWSADGLPNT	YNTIISRMGQ
130	140	150	160	170	180
WYIMIDICLG	YGKRKIHTVI	YDSLKKLPFP	VKKIAKDFKL	TVLKGDDIDYH	KERPVGYKIT
190	200	210	220	230	240
PEEYAYIKNA	IQIIAEALLI	QFKQGLDRMT	AGSDSLKGFK	DIITTKKFKK	VFPTLSLGLD
250	260	270	280	290	300
KEVRYAYRGG	FTWLNDRFKE	KEIGEGMVFD	VNSLYPAQMY	SRLLPYGEPI	VFEGKYVWDE
310	320	330	340	350	360
DYPLHIQHIR	CEFELKEGYI	PTTIQIKRSR	YKGNEYLKSS	GGEIADLWLS	NVDLELMKEH
370	380	390	400	410	420
YDLYNVEYIS	GLKFKATTGL	FKDFIDKWTY	IKTTSEGAIK	QLAKLMLNSL	YGKFASNPDV
430	440	450	460	470	480
TGKVPYLKEN	GALGFRLGEE	ETKDPVYTPM	GVFITAWARY	TTITAAQACY	DRIIYCDTDS
490	500	510	520	530	540
IHLTGTEIPD	VIKDIVDPKK	LGYWAHESTF	KRAKYLRQKT	YIQDIYMKEV	DGKLVEGSPD
550	560	570	580	590	
DYTDIKFSVK	CAGMTDKIKK	EVTFENFKVG	FSRKMMPKPV	QVPGGVVLVD	DTFTIK

(SEQ ID NO: 13)

[00466] In some embodiments, the biomolecule comprises a fusion protein having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence, which comprises a Phi-29 polymerase peptide that lacks exonuclease activity and comprises an N-terminal His-tag, an intervening linker sequence, and the D12A and D66A mutations, as follows:

MNHLVHHHHH	HIEGRHMELG	TLEGSMKHMP	RKMYSCAFET	TTKVEDCRVW	AYGYMNIEDH
70	80	90	100	110	120
SEYKIGNSLD	EFMAWVLKVQ	ADLYFHNLKF	AGAFIINWLE	RNGFKWSADG	LPNTYNTIIS
130	140	150	160	170	180
RMGQWYMIDI	CLGYKGKRI	HTVIYDSLKK	LPFPVKKIAK	DFKLTVLKGD	IDYHKERPVG
190	200	210	220	230	240
YKITPEEYAY	IKNDIQIIAE	ALLIQFKQGL	DRMTAGSDSL	KGFKDIITTK	KFKKVFP TLS
250	260	270	280	290	300
LGLDKEVRYA	YRGGFTWLND	RFKEKEIGEG	MVFDVNSLYP	AQMYSRLLPY	GEPIVFEGKY
310	320	330	340	350	360
VWDEDYPLHI	QHIRCEFELK	EGYIPTIQIK	RSRFYKGNEY	LKSSSGGEIAD	LWLSNVDLEL
370	380	390	400	410	420
MKEHYDLYNV	EYISGLKFKFA	TTGLFKDFID	KWTYIKTTSE	GAIKQLAKLM	LNSLYGKFAS
430	440	450	460	470	480
NPDVTGKVPY	LKENGALGFR	LGEEETKDPV	YTPMGVFITA	WARYTTITAA	QACYDRIIYC
490	500	510	520	530	540
DTDSIHLTGT	EIPDVIKDIV	DPKKLGYWAH	ESTFKRAKYL	RQKTYIQDIY	MKEVDGKLVE
550	560	570	580	590	600
GSPDDYTDIK	FSVKCAGMTD	KIKKEVTFEN	FKVGF SRKMK	PKPVQVPGGV	VLVDDTFTIK

(SEQ ID NO: 14)

[00467] This fusion polymerase of amino acid sequence of SEQ ID NO: 14 is herein variously referred to as “HP1” or “HP-1”. See, e.g., U.S. Provisional Application No. 61/184,770, filed June 5, 2009. This fusion polymerase comprises a Phi-29 polymerase peptide that lacks exonuclease activity and comprises an N-terminal His-tag, an intervening linker sequence, and the D12A and D66A mutations.

[00468] In some embodiments, the biomolecule comprises a fusion protein having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 14 (HP-1).

[00469] In some embodiments, a naturally occurring or engineered cysteine of SEQ ID NO: 14 (HP1) is used as an attachment site for a label. In some embodiments, the attachment site is a site for covalent attachment of the label using the linking agent SMCC. In some embodiments, the cysteine occurring at amino acid position 473 of SEQ ID NO: 14 (HP1) is used as the attachment site.

[00470] In some embodiments, the polymerase can comprise a His-tagged version of a Phi-29 polymerase and an N-terminal linker as well as various mutations that reduce the exonuclease activity of the Phi-29 polymerase.

[00471] In some embodiments, the polymerase, or any biologically active fragment thereof, can be linked to a label through a peptide linker comprising a series of amino acid residues. In some embodiments, the peptide linker comprises the amino acid sequence of SEQ ID NO: 12 or SEQ ID NO: 13.

[00472] In some embodiments, polymerase of the labeled polymerase conjugate comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 14.

[00473] In some embodiments, the polymerase comprises mutated Phi-29 DNA polymerase that lacks 3' to 5' exonuclease activity. In some embodiments, the mutant Phi-29 DNA polymerase comprises the mutations D12A, D66A, the mutation D169A, the mutation H61R, the mutation N62D, the mutation Q380A, the mutation S388G, or any combination thereof, wherein the numbering is relative to wild-type Phi-29 polymerase (SEQ ID NO: 3). In some embodiments, the polymerase comprises any two, three, four, five or all of these mutations.

[00474] In some embodiments, the polymerase falls with the family of Family type-B delta polymerases, the Type B DNA polymerases, or the Family A T7 like polymerases.

[00475] In some embodiments, the polymerase is capable of withstanding exposure to laser irradiation and/or exposure to labels for a duration of at least 5, 10, 15, 20, 30, 45, 60, 90, 120, 180 or 240 minutes. Without being bound to any particular theory, it is believed that the polymerases of phototrophic and/or halotrophic organisms can exhibit enhanced tolerance to laser irradiation, fluorescent dyes, nanoparticles, photo-breakdown products and/or excited state molecules such as superoxides, triplet oxygen, peroxides, etc. In some embodiments, the polymerase can be, for example, a polymerase isolated from a phototrophic and/or halotrophic organism. The polymerase can be a polymerase isolated from Cyanophage S-CBP1, Cyanophage S-CBP2, Cyanophage S-CBP3, Cyanophage Syn5, Cyanophage S-CBP42, Synechococcus phage P60, Roseobacter phage SIO1 DNA Polymerase, Oedogonium cardiacum chloroplast DNA Polymerase, Salterprovirus His1 Polymerase, Salterprovirus His2 Polymerase, Ostreococcus tauri V5, Ectocarpus siliculosus virus 1, or any combination of such polymerases.

[00476] In some embodiments, the biomolecule of the conjugate can be a Cyanophage S-CBP1 DNA polymerase having the following sequence:

```

1 mtlifdietd glyndascih cigihdl nag etyvfvndvgt qqpitkgiql ledadlivgh
61 niigydi pvi sklfpwfsrt ngvldtlvls rlyhtdlldi dqkrkwkhmp lqlygrhsle
121 aygyrlgeyk gsfgktadwk ewsqdm edym iqdvnvtrkl wkhfpqipew vql ehrvaqi
181 lteqei ygwy fd ena arela qtlytelddl kgv lrkrypy vagreftpkr vn rslgyveg
241 atctklvefs ptsrdhiawv mknlhgw kpd kktkagktai deivlkeigt eealqffrcl
301 eitkqlgmls egknawlkls rkdrvh hcs vatvthrc ah rnpnlaqvps dlnfr rlfca
361 spghimvgad lsgielrmla hylaryddgr ygdillhgdi hgenadkigi sr rlvktvty
421 aflygagd qk iglsydqgls pdkakqkgke irqaymdaip gleklveatk kaadrgfirs
481 idgrhin vds shkalnml lq ssagciakrw mviandnfpt idneylahth qlafihdelq
541 fec lplyaed lkthlelcae lageyy nlr i piaaegkigs twadvh

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(SEQ ID NO: 15)

[00477] In some embodiments, the biomolecule can comprise a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment

thereof. In some embodiments, the polymerase comprises a mutant or variant of Cyanophage S-CBP1 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00478] In some embodiments, the biomolecule of the conjugate can be a Cyanophage S-CBP2 DNA polymerase. In some embodiments, this DNA polymerase can have the following sequence:

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1 mklvfdietd gflrklttvh cvvakdietg evfkfddsgr hqsvssgltl lmeaeelwgh
61 niigfdvpai qeiyppffqpw estyydtlil srlfftdmld rdlrskpanm pgnlygrhsl
121 eawgyrlgvl kseygkqlhg dwatytpeml eyceqdvean lpivklfqpkl leqyadaikt
181 ehdcalvmtr qeqagfpfdi dkaraleskl rseletlsde mratftfvag keftparnna
241 trgyitgcpf tkltefspts rdhiawafqq hrgwepiemt dtgkpkidee vlnaigteea
301 kkfgrilelq khvgmlsegk nswlqmvekd grihhscvln tatgrnahmr pnlaqvpsgh
361 efrelftpgg gyvqvvgadas glelrclahy larfdggkfg kvlllegdiht dlaniygt dr
421 ktgktvtycl iygggdtklg lsagepkksa asrgkkirqa imkdldgfaq litavqeraq
481 sgvitgidgr pirmrkahaa lnyllqscga vickkwvvr nellteagid ytplafvhde
541 qqlavrpdpv emastlisl mkdvehaikf rvpldcdvqs ganwgdth

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(SEQ ID NO: 16)

[00479] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Cyanophage S-CBP2 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00480] In some embodiments, the biomolecule of the conjugate can be a Cyanophage S-CBP3 DNA polymerase. In some embodiments, the DNA polymerase can have the following sequence:

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1 mtlifdietd glyndvtcih cigihdlnk etyvfndvgt qqpitkgiql ledadiivgh

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61 niigydlpvi rklypwfsnv grvldtlvls rlyhadllkt dqkrnwkhmp vqlwgrhsle
121 aygyrlgeyk gcfgktttdwk dwsqemadym vqdvnitrl wkdfpeipew vqllehrvaqi
181 lteqei hgwy fdepaawe le stlrrelesl kavlrnrhpf ilgeeftpkr pnstqgyftg
241 atftrlkemn ptsrdhiayi lqkfydwept ertekgkpvv deivlkdigs eialqffrcl
301 eltkqigmlt egvnawklv rndrihhhs vatnthrcah rkpnlaqvpa eaefrklfra
361 tpgmvmvgad lagielrmla hylaqwdggr ygdvllngdi hqenackigi srllvktvty
421 aflygagnqk iglsyds sls pdkakkkgqe irqaymdaip glrklveatk kaanrgyira
481 idgrhisvds phkslnyllq ssagviakrw laltheaiir adikahqlaf ihdelqfett
541 pehvedlkfa llwgaasage yynlripiaa daksgndwse vh

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(SEQ ID NO: 17)

[00481] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Cyanophage S-CBP3 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00482] In some embodiments, the biomolecule of the conjugate can be a Cyanophage Syn5 DNA polymerase. In some embodiments, the DNA polymerase can have the following sequence:

```

1 mrlvfdietd gllrglsvih civardldtn eehrfephqt kagqlllkea delwghnivg
61 ydieaikely pkwttkakly dtlilsrlff tdlldrdf rs kpanmpgnly grhsleawgh
121 rlgvhksefg kqldgdwsty spemleycaq dvtvsvqvaq mfepkleqya dcidtehrla
181 timawqereg fpfdvtaaq lesrlrteld alsdqmrstf lfvdggftft rrrnkpqgyi
241 adapmcklke fnptsrhhia wafqqfrnwe pkeftdsgkp kideptltai gtdeakafar
301 ilelqkhlqg laegknawlk leskgrvhhs cvlntntgrq ahmrpnlaqv psaseyralf
361 gpgdsrvqvg adasglelrc lahylapfdn gsfaetvng dihtelasiy gtdrksgkgv
421 tycliygggd hklgstagas kaqaskkgke irgrimrdld gfaalsdavs rrartgvrlg
481 ldgrpirllq kshaalnyll qsagavickq wllrsyelld eanidywpla fvhdelqisv
541 apsqaeat1 litaamkdvq hnlkfrceld seaqtgns wa dch

```

(SEQ ID NO: 18)

[00483] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Cyanophage Syn5 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00484] In some embodiments, the biomolecule of the conjugate can be a Cyanophage S-CBP42 DNA polymerase. In some embodiments, the DNA polymerase can have the following sequence:

```

1 mrlafdietd gllrnltkih civaqldtn evykfdgtgd hpsireglal lkdadelwgh
61 niigydfear kevfpwnys stvydtlils rlfftdlldr dfrsrpanmp aqlygrhsle
121 awghrlsvhk sefgkslsgd wstyspemld ycardvvsv slarlftakv aeyrdciste
181 hrlatimawq esegfpfdva kaerlegqlr sellklseqm retfpyvdgg sftprtnngp
241 rgyvkgaaac rlkefnptsr qhiawafatf rdwepkeltd tgkpkidett lleygtdeak
301 tfarilelqk hlgqlsega awlkkvesdg rihhscvlnt ntgrqahmkp nlaqvpsghe
361 yrelfhpgan rsqvgadasg lelrcighyl arfdggkfak evvqgdihta laeiygtdrk
421 sgkgvtycli ygggdsklgl tagaskaqav kkgkeirsri manldgfaal naavqeraks
481 gvlkgldgrp irlqgnhaa lnyllqsaga vicklwllrs yelldeagid yfpmafvhde
541 vhisvapsqa eqagqliqia mkdvehqikf rcaldseyqi gnswadch

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(SEQ ID NO: 19)

[00485] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Cyanophage S-CBP42 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00486] In some embodiments, the biomolecule of the conjugate can be a *Synechococcus* phage P60 DNA polymerase. In some embodiments, the DNA polymerase can have the following sequence:

```

1 mklafdietd glipdltihi civardidtd eefrfdgtgd ypsikeglel lskadelwgh
61 nivnydypai qklhpdwtp sctrdtlils rlfftdlldr dfrsrpalmp gnlygrhsle
121 awghrlghhk sefgkslegd wstyspemle ycardvevsv alaktfvpki peyqwsvdte
181 heiarimswq eqmgfvpdvr aaqalegklr leldtlddm retfhdvdgg vmtpkrsnk
241 rhyfenapfc klrefnptsr hhiawafehh rgwepkerta ggqpkiddei lreintkesl
301 afarilelqk hlgqlsegkn awlklerkgr lhhscvlnn tgrqahmrpn laqvpsahey
361 rslfkpsdnh lqvgsdasgl elrcldghyls rydggkfdae vvnngdihtal aeiygtdrks
421 gkgvtycliy ggnhklglgt agaskssasr kgqeirgkim qglsgfadln aaiqeraksg
481 vlkgldgrpi rlqgknhaal nylqsagai icklwirth ellqeagidy yplafvhdeq
541 qlsvradqae maaqlttlam kdvehqvkrf caldseyqig nswadch

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(SEQ ID NO: 20)

[00487] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of *Synechococcus* phage P60 DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00488] In some embodiments, the biomolecule of the conjugate can be a *Roseobacter* phage SIO1 DNA polymerase. In some embodiments, the *Roseobacter* phage SIO1 DNA polymerase can have the following sequence:

```

1 mevvdietd aldatvihvl vakrvqkgf yvvrdaetfk rlakqvtlwi ghnvigfdip
61 qikkllwgygi plkdvadtlv msrllldptrk gghsldalsg nekidfhdfs tytpemlayc
121 kqdvainekyl qlqlkeelsn fgkasiqlh qmqaivceqe kngfmltdi aeeyttclr
181 etnrieaeik efmvpiavpv keviikrkkd gsiysnqlle gcnvqgdytk iaweefnlgs
241 paqvnkrldr lgwkptvktk sgnsykicpe nlatipdtap eavkglkawv vletrwklaq
301 ewlqksqetg rvhgrviltg avthraahqg pnmanipsvp hgkdgilwkm egmygaecrq
361 afkvpegkll vgtdaagiql rvlahymndp iyteqvidgd ihtfnkealg ryckdrptak
421 tfiyafllga gtgmiasilg cnnrqaneam anfyaeipsl kklksqasqa asmgwmkgld
481 grvlrigsdh lalsvylqgg etvimrlanv fwqrqakkeg infkqcawvh dewqtevded

```

541 qaqrllgeiqv qaikdagtff klncpmdgea kigknwleth

(SEQ ID NO: 21)

[00489] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Roseobacter phage SIO1 DNA Polymerase DNA polymerase that lacks 3' to 5' exonuclease activity. For example, the so-called "DIET" motif comprising the amino acid residues DIET from positions 6-9 of the above amino acid sequence can be mutated via substitution of both the Asp and Glu residues of the DIET motif with Alanine, resulting in a polymerase that lacks 3' to 5' exonuclease activity.

[00490] In some embodiments, the biomolecule of the conjugate can be a DNA polymerase from Oedogonium cardiacum. In some embodiments, the Oedogonium cardiacum polymerase can be a Oedogonium cardiacum chloroplast DNA Polymerase. In some embodiments, this polymerase can have the following sequence:

1 miefyasfdk dkeieinked semnkediem nkedieidld evneeerfdv nremlqtnyf
 61 vkrfknilfp iaasfytseg nknvsktfs1 tsnifdkkip stinilkesq immqefliel
 121 islaedllkk rnptnslfyg ddkviiymhn lssfdgffil qtlkksrln ytfnlknkklk
 181 vtsyegliyr ikignlcfqd syrvipmsln klsflllnkq kkdfdvenin sqklqhifkn
 241 keilekmley clydsillye smiliqktfw delkfditse stisntainf ffskyyefpt
 301 qyywhtttkk dglsaklkyl nkrvtvsthh naifytkpfl dqqlrsayfg grtelykpgt
 361 sngyvfdins lyafalmydm pygspiyene yknwttnefe sffgflkiif itppnydilp
 421 vlprypppi shnvyclgig egwyfseeik larqgyklk ilesikftph kgfekfvrdp
 481 fsirqqypkg hplnllakli lnstygrfgi altthkqmkf fnqiklkekk nkkinini

(SEQ ID NO: 22)

[00491] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Oedogonium cardiacum chloroplast DNA Polymerase DNA polymerase that lacks 3' to 5' exonuclease activity.

[00492] In some embodiments, the biomolecule of the conjugate can be a Salterprovirus His1 polymerase. In some embodiments, the polymerase can have the following sequence:

```

1 makcdkslea idldraytap rkakwaenkr ingldtetsd gdifcisvcw egekpmvqhn
61 drekltskqv wqvltdhkar sslnmwynld fdanvvlnehv cseeqlaelv vsqttlansd
121 rtyrqymdtd kelrkgeyli tyiqskflei kdhnsihyth ydasqffyts lenavtewlg
181 eskandglea glfgsqtpnq lretvaesdc vtwnlslty nvskgdkwti hnaksyiskn
241 wsdilkyaqi daelvrldwq eavnvgeeld ipmgrpfstg ylaesyldnr lrekpplgpm
301 pmakmawesy aggrfevlkr gnvgrvagpd insaypavla elpdpktrlw krakhasise
361 ietadygfmt vkvstdptre iqpfavkdek qdklvypspq nteitvvkdi fihaynqgyv
421 tdyevidcwl gyktegttftp fdfipelydn rktaeangle krglllkiivl nsmygktcqt
481 tpkrrelaes telelhesyv pdmslpkmir ekysegfies ltagawfnpf lasyitgltr
541 lelhkqickh dleentvmla tdcvmieekp feesnfvenl vqdglygwdm eykgdafvlg
601 agvyqidfdt cqkgckdncn kfshkhkvkt rgfseadlek glvnaaekan ghieiestrp
661 qtiseiiswn eelsqvgnfl egerkikpem dtkrkwsent dfkklstce tslplki

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(SEQ ID NO: 23)

[00493] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Salterprovirus His1 DNA polymerase that lacks 3' to 5' exonuclease activity.

[00494] In some embodiments, the biomolecule of the conjugate can be a DNA polymerase from Salterprovirus His2. In some embodiments, this polymerase can have the following amino acid sequence:

```

1 maksdrnlde vnlypayqdq ysatfvdgkl inafdtetss gtvfmltsay gdktqayynr
61 dvseldaeti mdaltdyctr sniniwynld fdanailsgi lsqkemselv vtneffffffa
121 gieyeifyik gkmlrivden gnisphydia qffytstlnd aewlgenkk egidtskfdd
181 keyikdnfde ilkyakkdas ltqdlaielt neaenldipm grpistgyls aeylrantee
241 kpslgneamq nlfwesyygg rfevfqrgnv gevvpdins aypaimkdlp dpttlnwnhy
301 lnevsdkepf shsinkfgye eienghygvv karvttssr miqpfackid gkvkfpamtn
361 kvvtvikpif efavnnglvt dfelieawig nitdrtskpf efigdmyaer kvfeqlknkp
421 kkgqlkivl nssygktcqt tekrrkhld kdgkkimqah etqyprfysl kkqrealgdd
481 eiiiiteleag krfnppfasy itgltrlelh kvvvehdied stvmfatdcl mvekeayens
541 sfdeqihvpd dslpesefrk eatrslgawd fdyegsafiv gsgvyevdti qgkttktrg
601 fiesnlgdtl kglakkkhea ipldnerplt maevlinter gsvsefvns kklkpdfddk

```

661 rnwnrenpnf hdlldkeys kpidlqeqke emiqeqmdin ekmigdatpn gnetvvvkdd

(SEQ ID NO: 24)

[00495] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of Salterprovirus His2 DNA polymerase that lacks 3' to 5' exonuclease activity.

[00496] In some embodiments, the biomolecule of the conjugate can be a DNA polymerase from *Ostreococcus tauri* V5. In some embodiments, this DNA polymerase can have the following amino acid sequence:

```

1 mvvfqaltwe srdtddehli sifgkteegk svclttaftp yffiklpeki dagkirriyn
61 ildekckdsl vaysvmkskd vwgfqneef vfmkvnfkh1 qarlvdsfl rkpldrtpel
121 fnifgvrnvk vyesnldpvl rlmhrtgiqs tgwldtgdke irshlarvdl dlfendwttl
181 kpvarddiap fvvasvdiec nsstgkfpda dvtgdacfqi aislckfgsd epydktclcy
241 kktdpnlegs tirsyetere mleafqkylh tkdvditgw nifgfdmeyi ykraqvnrch
301 yeffnlgklr dteselvikk lsssalgdn1 lkllpmpgrf ifdmfhevkk gykldsykld
361 nvsklylgdq kidmapkemf aryreedpvk lrevaeycik dtllphrlmk klctllnmve
421 makatwvpan flvergqgik vfsqtkkar elgfmvptir ygaipeepye gatvleaqkg
481 ayytpitald fealypsimm ahnlyssyv mdekrygsvp gityetfnig drtykfaqdv
541 psllpailae lkqfrkqakr dmaaatgfmk evyngkqlay kvsmnsvygf tgagkgilpc
601 vpiasttttsk grsmieetkn yveknfpgak vrygdtsvm vefdvgrkg eeaiayswev
661 geraaeecsa lfkkpnnlel ekvywpyfly skkryaaklw tkgkdgkmhm dyidikglqv
721 vrrdntphvr evckelldvi ltssdpqppk elakeraiel lsgdvpndkl ilsqglstdy
781 kvggknsvst sadsvninqs hvqvvtkmrq rkpgsepqsg drvpylltkt qdpkakeyek
841 aedpkyveeh gvpvdyhyf lnkflnpvcd lldplyenvk edifgeiina hkpvkppklp
901 slsgmkkddl iaecqrlgle etgtlailra rlkdarhgsv edlfknyelt qskdss

```

(SEQ ID NO: 25)

[00497] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of *Ostreococcus tauri* V5 DNA polymerase that lacks 3' to 5' exonuclease activity.

[00498] In some embodiments, the biomolecule of the conjugate can be a DNA polymerase from *Ectocarpus siliculosus* virus 1. In some embodiments, this polymerase can have the following amino acid sequence:

```

1  melylhdir d nsgsfqnptm qlfameedgt nvfvsvknfk tylyvgfdld isedsvrsny
61  lekfkqekwe rnvykmsvkv rkrligfsng dlfpylmef tgtisfyivr khlheloger
121 dpgpntfvd l nkypgmcvye sksvdsilkf fhasgvrpss yfrmenyvr v adkarkthca
181 kefivdfvnv rpvgeevvdr kpppmticsy dletsglntn edyifqasmi fsrlgdpcpd
241 segsatghav dsytdgvvic vgdtesvdgt pllivenelq lldkfreilv ergcnilcgy
301 ntfkfdsafl ykraerygfd gfkklsfikd lacdlevctl qsaalgknel kqiipgrve
361 idlfmvmrrs qklssykl na vcdkffggkk ddvtyadilq actskdpkkl gviakycyqd
421 sglvklldk ikevdatem akltcvplty ivgrgqqikc mslilnrihg eyvcnyaaak
481 kkmaadgkqv lnegykgasv idakkgyek dpivtmdfas lypsimrlkq lcyttivrdv
541 kyrgiegvny edhqisdgvs vtfahrpgsr silceleeml geerkatkkk mksekdpfay
601 slldskqkaq kvtmnsiygf tgtvnngmlp lveiaaavts tgrdmikrtk eyaekehgc n
661 viygdtdsvm vifpehrnie nlgdkmrycf dmgtkvskei semfghpill efeniyfkyl
721 lvskkryagl swetveg pmtmkgltvr rdnafvgrc aseaihmlmd vdvtdgrgav
781 kkhl tetllr lergqisied ltirkelkqw vyktpsphat lalkilertk eqavfrefik
841 payetiggyd dslssvwtk mtnlksylsv rakreiamsd mvesirgdt spfkaeayav
901 valrqlyddv hsvlvgesfa rvvglvmagi gdvhklgery mafvrynivd wdpptlgeri
961 pyvittgkgd issraedprm vnvgrcrpdf lyyidhqlrn pmvdllqhvi espsslfves
1021 qrrmsnl nhg rkeittffkk rkvteg

```

(SEQ ID NO: 26)

[00499] In some embodiments, the biomolecule can be a polymerase having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the above amino acid sequence, or any biologically active fragment thereof. In some embodiments, the polymerase comprises a mutant or variant of *Ectocarpus siliculosus* virus 1 DNA polymerase that lacks 3' to 5' exonuclease activity.

[00500] In some embodiments, the biomolecule comprises the amino acid sequence of SEQ ID NO: 14 (HP1), further comprising a mutation of one, some or all of the cysteines occurring at amino acid positions 47, 315, 473 and/or 555 to any other amino acid. In some embodiments, the cysteine is replaced with a serine or alanine residue. In some embodiments, the cysteines at amino acid positions 47, 315 and 555

are mutated to another residue, e.g., alanine or serine, and the cysteine at position 473 is not mutated and can serve as a site for covalent attachment of a label.

[00501] In some embodiments, the biomolecule comprises the amino acid sequence of SEQ ID NO: 14 (HP1) and further comprises the mutation Q380A:

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>	<u>60</u>
MNHLVHHHHH	HIEGRHMELG	TLEGSMKHMP	RKMYSCAFET	TTKVEDCRVW	AYGYMNIEDH
<u>70</u>	<u>80</u>	<u>90</u>	<u>100</u>	<u>110</u>	<u>120</u>
SEYKIGNSLD	EFMAWVLKVQ	ADLYFHNLFK	AGAFIINWLE	RNGFKWSADG	LPNTYNTIIS
<u>130</u>	<u>140</u>	<u>150</u>	<u>160</u>	<u>170</u>	<u>180</u>
RMGQWYMIDI	CLGYKGKRKI	HTVIYDSLKK	LPFPVKKIAK	DFKLTVLKGD	IDYHKERPVG
<u>190</u>	<u>200</u>	<u>210</u>	<u>220</u>	<u>230</u>	<u>240</u>
YKITPEEYAY	IKNDIQIIAE	ALLIQFKQGL	DRMTAGSDSL	KGFKDIITTK	KFKKVFPTLS
<u>250</u>	<u>260</u>	<u>270</u>	<u>280</u>	<u>290</u>	<u>300</u>
LGLDKEVRYA	YRGGFTWLND	RFKEKEIGEG	MVFDVNSLYP	AQMYSRLLPY	GEPVIFEGKY
<u>310</u>	<u>320</u>	<u>330</u>	<u>340</u>	<u>350</u>	<u>360</u>
VWDEDYPLHI	QHIRCEFELK	EGYIPTIPIK	RSRFYKGNEY	LKSSSGEADI	LWLSNVDLEL
<u>370</u>	<u>380</u>	<u>390</u>	<u>400</u>	<u>410</u>	<u>420</u>
MKEHYDLYNV	EYISGLKFKA	TTGLFKDFID	KWTYIKTTSE	GAIKALAKLM	LNSLYGKFAS
<u>430</u>	<u>440</u>	<u>450</u>	<u>460</u>	<u>470</u>	<u>480</u>
NPDVTGKVPY	LKENGALGFR	LGEETKDPV	YTPMGVFITA	WARYTTITAA	QACYDRIIYC
<u>490</u>	<u>500</u>	<u>510</u>	<u>520</u>	<u>530</u>	<u>540</u>
DTDSIHLTGT	EIPDVIKDIV	DPKKLGWYAH	ESTFKRAKYL	RQKTYIQDIY	MKEVDGKLVE
<u>550</u>	<u>560</u>	<u>570</u>	<u>580</u>	<u>590</u>	<u>600</u>
GSPDDYTDIK	FSVKCAGMTD	KIKKEVTFEN	FKVGFSRKMK	PKPVQVPGGV	VLVDDTFTIK

(SEQ ID NO: 27)

[00502] This fusion polymerase having the amino acid sequence of SEQ ID NO: 27 is hereinafter referred to as HP1 Q380A. In some embodiments, the biomolecule comprises a fusion protein having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 27.

[00503] In some embodiments, the biomolecule comprises the amino acid sequence of SEQ ID NO: 28, below, which is the HP1 sequence further comprising the mutation S388G:

```

      10      20      30      40      50      60
MNHLVHHHHH HIEGRHME LG TLEGSMKHMP RKMYS CAFET TTKVEDCRVW AYGYMNIEDH
      70      80      90     100     110     120
SEYKIGNSLD EFMAWVLKVQ ADLYFHN LKF AGAFIINWLE RNGFKWSADG LPNTYNTIIS
     130     140     150     160     170     180
RMGQWYMIDI CLGYKGKRKI HTVIYDSLKK LPFPVKKIAK DFKLTVLKGD IDYHKERPVG
     190     200     210     220     230     240
YKITPEEYAY IKNDIQIIAE ALLIQFKQGL DRMTAGSDSL KGFKDIITTK KFKKVFP TLS
     250     260     270     280     290     300
LGLDKEVRYA YRGGFTWLND RFKEKEIGEG MVFDVNSLYP AQMYSRLLPY GEPIVFEGKY
     310     320     330     340     350     360
VWDEDYPLHI QHIRCEFELK EGYIPTIQIK RSRFYKGNEY LKSSGGEIAD LWLSNVDLEL
     370     380     390     400     410     420
MKEHYDLYNV EYISGLK FKA TTGLFKDFID KWTYIKTTSE GAIKQLAKLM LNGLYGKFAS
     430     440     450     460     470     480
NPDVTGKVPY LKENGALGFR LGEEETKDPV YTPMGVFITA WARYTTITAA QACYDRIIYC
     490     500     510     520     530     540
DTDSIHLTGT EIPDVIKDIV DPKKLG YWAH ESTFKRAKYL RQKTYIQDIY MKEVDGKLVE
     550     560     570     580     590     600
GSPDDYTDIK FSVKCAGMTD KIKKEVTFEN FKVGF SRKMK PKPVQVPGGV VLVDDTFTIK

```

(SEQ ID NO: 28)

[00504] This fusion polymerase of amino acid sequence of SEQ ID NO: 28 is herein referred to as HP1 S388G. In some embodiments, the biomolecule comprises a fusion protein having an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 28 (HP1 S388G).

[00505] In some embodiments, the biomolecule is a His-tagged version of a polymerase isolated from the phage RB69. The His-tag can be fused to the N-terminus, the C-terminus or any other suitable position of the RB69 polymerase. Optionally, the His-tag can be separated from the amino acid residues of the protein by a linker, typically a peptide linker. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

```

      10      20      30      40      50      60
MHHHHHHKHM KEFYLTVEQI GDSIFERYID SNGRERTREV EYKPSLFAHC PESQATKYFD
      70      80      90     100     110     120

```

```

IYGKPTRKL FANMRDASQW IKRMEDIGLE ALGMDDFKLA YLSDTYNYEI KYDHTKIRVA
  130      140      150      160      170      180
NFDIEVTSPD GFPEPSQAKH PIDAITHYDS IDDRFYVFDL LNSPYGNVEE WSIEIAAKLQ
  190      200      210      220      230      240
EQGGDEVPS EIDKIIYMPF DNEKELLMEY LNFQQKTPV ILTGWNVESF DIPYVYNRK
  250      260      270      280      290      300
NIFGESTAKR LSPHRKTRVK VIENMYGSRE IITLFGISVL DYIDLYKKFS FTNQPSYSLD
  310      320      330      340      350      360
YISEFELNVG KLKYDGPISK LRESNHQRYI SYNIIDVYRV LQIDAKRQFI NLSLDMGYA
  370      380      390      400      410      420
KIQIQSVFSP IKTWDALIFN SLKEQNKVIP QGRSHPVQPY PGAFVKEPIP NRYKYVMSFD
  430      440      450      460      470      480
LTSLYPSIIR QVNISPETIA GTFKVAPLHD YINAVAERPS DVYSCSPNGM MYYKDRDGVV
  490      500      510      520      530      540
PTEITKVFNQ RKEHKGMYLA AQRNGEIIKE ALHNPNSVD EPLDVEDYRFD FSDEIKEKIK
  550      560      570      580      590      600
KLSAKSLNEM LFRAQRTEVA GMTAQINRKL LINSLYGALG NVWFRYYDLR NATAITTFGQ
  610      620      630      640      650      660
MALQWIERKV NEYLNEVCGT EGEAFVLYGD TDSIYVSADK IIDKVGESKF RDTNHVWDFL
  670      680      690      700      710      720
DKFARERMEP AIDRGFREMC EYMNKQHL MFDREAIAGP PLGSKGIGGF WTGKKRYALN
  730      740      750      760      770      780
VWDMEGTRYA EPKLKIMGLE TQKSSTPKAV QKALKECIRR MLQEGEESLQ EYFKEFEKEF
  790      800      810      820      830      840
RQLNYISIAS VSSANNIAKY DVGGFPGPKC PFHIRGILTY NRAIKGNIDA PQVVEGEKVY
  850      860      870      880      890      900
VLPLREGNPF GDKCIAWPSG TEITDLIKDD VLHWMDYTVL LEKTFIKPLE GFTSAAKLDY
  910
EKKASLFDMF DF

```

(SEQ ID NO: 29)

[00506] In some embodiments, the biomolecule is a His-tagged version of a polymerase isolated from the GA-1 phage. The His-tag can be fused to the N-terminus, the C-terminus or any other suitable position of the GA-1 polymerase. Optionally, the His-tag can be separated from the amino acid residues of the protein by a linker. In some embodiments, the linker comprises the F-linker sequence LLGGGSGGGGSAAAGSAA (SEQ ID NO: 5). In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

```

      10      20      30      40      50      60
MHHHHHHKHM ARSVYVCDFE TTTDPEDCRL WAWGWMDIYN TDKWSYGEDI DSFMEWALNS
      70      80      90     100     110     120
NSDIYFHNLK FDGSFILPWW LRNGYVHTEE DRINTPKFT TTISGMGQWY AVDVCINTRG
     130     140     150     160     170     180
KNKNHVVFYD SLKKLPFKVE QIAKGFGLPV LKGDIDYKKY RPYGYVMDDN EIEYLKHDLL
     190     200     210     220     230     240
IVALALRSMF DNDFTSMTVG SDALNTYKEM LGVKQWEKYF PVLSLKVNSE IRKAYKGGFT
     250     260     270     280     290     300
WVNPKYQGET VYGGMVFDVN SMYPAMMKNK LLPYGEPMVF KGEYKKNVEY PLYIQQVRCF
     310     320     330     340     350     360
FELKKDKIPC IQIKGNARFG QNEYLSTSGD EYVDLYVTNV DWELIKKHYD IFEEEFIGGF
     370     380     390     400     410     420
MFKGFIGGFD EYIDRFMEIK NSPDSSAEQS LQAKMLNSL YGKFATNPDI TGKVPYLDEN
     430     440     450     460     470     480
GVLKFRKGEL KERDPVYTPM GCFITAYARE NILSNAQKLY PRFIYADTDS IHVEGLGEVD
     490     500     510     520     530     540
AIKDVIDPKK LGYWDHEATF QRARYVRQKT YFIETTWKEN DKGKLVVCEP QDATKVKPKI
     550     560     570     580
ACAGMSDAIK ERIRFNEFKI GYSTHGSLKP KNLGGVVLN DYPFAIK

```

(SEQ ID NO: 30)

[00507] In some embodiments, the biomolecule is a His-tagged version of a polymerase isolated from the B103 phage. The His-tag can be fused to the N-terminus, the C-terminus or any other suitable position of the B103 polymerase. Optionally, the His-tag can be separated from the amino acid residues of the protein by a linker. In some embodiments, the linker comprises the amino acid sequence LLGGGGSGGGGSAAAGSAA (SEQ ID NO: 5). In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

```

      10      20      30      40      50      60
MHHHHHHKHM PRKMFSCDFE TTTKLDDCRV WAYGYMEIGN LDNYKIGNSL DEFMQWVMEI
      70      80      90     100     110     120
QADLYFHNLK FDGAFIGVNW EHHGFKWSNE GLPNTYNTII SKMGQWYMID ICFGYKGKRR
     130     140     150     160     170     180
LHTVIYDSLK KLFPVVKKIA KDFQLPLLKG DIDYHAERPV GHEITPEEYE YIKNDIEIIA
     190     200     210     220     230     240
RALDIQFKQG LDRMTAGSDS LKGFKDILST KKFNVKVPKL SLPMDKEIRR AYRGGFTWLN
     250     260     270     280     290     300
DKYKEKEIGE GMVFDVNSLY PSQMYSRPLP YGAPIVFQGK YEKDEQYPLY IQRIRFEFEL
     310     320     330     340     350     360
KEGYIPTIQI KKNPFFKGNE YLKNSGAEPV ELYLTNVDLE LIQEHYEMYN VEYIDGFKFR
     370     380     390     400     410     420

```

EKTGLFKEFI	DKWTVKTHE	KGAKKQLAKL	MFDSLKGKFA	SNPDVTGKVP	YLKEDGSLGF
430	440	450	460	470	480
RVGDDEYKDP	VYTPMGVFIT	AWARFTTITA	AQACYDRIIY	CDTDSIHLTG	TEVPEIIKDI
490	500	510	520	530	540
VDPKKLGYWA	HESTFKRAKY	LRQKTYIQDI	YAKEVDGKLI	ECSPEDEATTI	KFSVKCAGMT
550	560		570		580
DTIKKKVTFD	NFRVGFSSSTG	KPKPVQVNGG	VVLVDSVFTI	K	

(SEQ ID NO: 31)

[00508] In some embodiments, the polymerase of the labeled polymerase conjugate is derived from a DNA polymerase of the Phi-29-like phage B103. The genome of B103, including a gene encoding a B103 DNA polymerase, has been sequenced. See, e.g., Pecenkova et al., "Bacteriophage B103: complete DNA sequence of its genome and relationship to other *Bacillus* phages" *Gene* 199:157-163 (1999). The DNA polymerase of B103 is homologous to the DNA polymerase of Phi-29 and of other Phi-29-like phages. Collectively, these polymerases share several highly conserved regions. See, e.g., Meijer et al., "Phi-29 family of phages" *Microbiol. & Mol. Biol. Revs.* 65(2):261-287 (2001). These conserved regions are typically characterized by several conserved amino acid motifs. See, e.g., Blanco et al., *Gene* 100:27-38 (1991); Blasco et al., "Φ29 DNA polymerase Active Site" *J. Biol. Chem.* 268:16763-16770 (1993) (describing regions of sequence homology and mutational analysis of consensus regions of Phi-29 and Phi-29-like DNA polymerases); Berman et al., "Structures of phi29 DNA polymerase complexed with substrate: the mechanism of translocation in B-family polymerases", *EMBO J.*, 26:3494-3505 (2007). Site-directed mutagenesis indicates that these three regions can form an evolutionarily conserved polymerase active site.

[00509] In some embodiments, the polymerase of the labeled polymerase conjugate is derived from a B103 polymerase comprising the amino acid sequence of SEQ ID NO: 32 as follows:

```

1   mprkmfscdf etttklddcr vwaygyeig nldnykigns ldefmqwvme iqadlyfhnl
61  kfdgafivnw lehngfkwsn eglpntynti iskmqgwymi dicfgykgkr klhtviyds1
121 kklpfpvkki akdfqlpllk gdidyaerp vgheitpeey eyikndieii araldiqlfkq
181 glrmtagsd slkgfkdi1s tdkfnkvfpk lslpmdkeir rayrggftwl ndkykekeig
241 egmvfdvns1 ypsqmysrpl pygapivfqq kyekdeqypl yiqrirfefe lkegyiptiq
301 ikknppffkgn eylnksaep velyltnvdl eliqehyemy nveyidgfkf rektglfkef
361 idkwtyvkth ekgakkqlak lmfdslygkf asnpdvtgkv pylkedgslg frvgdeeykd

```

421 pvytpmgvfi tawarfttit aaqacydrii ycdtdsihlt gtevpeiikd ivdpkklgyw
481 ahestfkrak ylrqktyiqd iyakevdgkl iecspdeatt tkfsvkcagm tdtikkkvtf
541 dnfrvgfsst gkpkpvqvng gvvlvdsvft ik

(SEQ ID NO: 32)

[00510] In some embodiments, the polymerase of the labeled polymerase conjugate is a variant of a B103 polymerase, wherein the modified polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 32, or any biologically active fragment thereof.

[00511] In some embodiments, the polymerase of the labeled polymerase conjugate is homologous to a polymerase of one or more of the following organisms: B103, Phi-29, GA-1, PZA, Phi-15, BS32, M2Y, Nf, G1, Cp-1, PRD1, PZE, SF5, Cp-5, Cp-7, PR4, PR5, PR722, or L17. See, e.g., Meijer et al., "Phi-29 family of phages," Microbiol. & Mol. Biol. Revs. 65(2):261-287 (2001).

[00512] In some embodiments, the polymerase of the labeled polymerase conjugate comprises a B103 polymerase having the amino acid sequence of SEQ ID NO: 32 and further comprises one or more mutations in the amino acid sequence of SEQ ID NO: 32. In some embodiments, the one or more mutations can include, for example, substitution, chemical modification, addition, deletion and/or inversion of one or more amino acid residues, or any combination of the foregoing.

[00513] The mutant B103 polymerase can optionally further comprise the amino acid sequence of any of the polymerases disclosed in U.S. Serial No. 61/242,771, filed on September 15, 2009; U.S. Serial No. 61/293,618, filed on January 8, 2010 or U.S. Serial No. ___ titled "Polymerase Compositions & Methods", Attorney Docket No. LT00052, filed concurrently herewith.

[00514] In some embodiments, the polymerase of the labeled polymerase conjugate comprises an amino acid modification at position 383, at position 384, or at both positions 383 and 384, wherein the numbering is relative to a B103 polymerase having the amino acid sequence of SEQ ID NO: 32. The modification can include, for example, one or more amino acid substitutions, additions, deletions or chemical modifications.

[00515] In some embodiments, the polymerase of the labeled polymerase conjugate is a variant of a B103 polymerase comprising the amino acid sequence of SEQ ID NO: 32, wherein the modified polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 32, or any biologically active fragment thereof, wherein the amino acid at position 383 is not phenylalanine (F), where the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00516] In some embodiments, the polymerase of the labeled polymerase conjugate comprises the amino acid sequence of SEQ ID NO: 32, and further comprises an amino acid substitution at position 383, wherein the numbering is relative to a B103 polymerase having the amino acid sequence of SEQ ID NO: 32. In some embodiments, the modified polymerase is a variant of B103 polymerase that comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 32, or any biologically active fragment thereof, wherein the modified polymerase further comprises the amino acid mutation F383L.

[00517] In some embodiments, the polymerase of the labeled polymerase conjugate is a variant of a B103 polymerase that comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 32, or any biologically active fragment thereof, wherein the amino acid at position 384 is not aspartic acid (D), where the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00518] In some embodiments, the polymerase of the labeled polymerase conjugate comprises the amino acid sequence of SEQ ID NO: 32 and further comprises an amino acid substitution at position 384, wherein the numbering is relative to a B103 polymerase having the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase is a variant of B103 polymerase that comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 32, or any biologically active fragment thereof, wherein the modified polymerase further comprises the amino acid mutation D384N.

[00519] In some embodiments, the polymerase of the labeled polymerase conjugate is a variant of B103 polymerase, or any biologically active fragment thereof, having the amino acid sequence of SEQ ID NO: 32, wherein the variant further comprises amino acid substitutions at positions 383 and 384, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises the amino acid sequence of SEQ ID NO: 32 and further comprises the amino acid substitutions F383L and D384N, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. The amino acid sequence of this polymerase can be represented as follows:

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1  mprkmfscdf etttklddcr vwaygyeig nldnykigns ldefmqwvme iqadlyfhnl
61  kfdgafivnw lehngfkwsn eglpntynti iskmqgwy mi dicfgykgkr klhtviyds1
121 kklpfpvkki akdfqlpllk gdidyaerp vgheitpeey eyikndieii araldiqfkq
181 glrmtagsd slkgfk dils t k k f n k v f p k l s l p m d k e i r r a y r g g f t w l n d k y k e k e i g
241 egmvfdvns1 ypsqmysrpl pygapivfqq kyekdeqypl yiqrirfefe lkegyiptiq
301 ikknppffkgn eylknsgaep velyltnvdl eliqehyemy nveyidgfkf rektglfkef
361 idkwtyvkth ekgakkqlak lmlnslygkf asnpdvtgkv pylkedgslg frvgdeeykd
421 pvytpmgvfi tawarfttit aagacydrii ycdtdsihlt gtevpeiikd ivdpkklgyw
481 ahestfkrak ylrqktyiqd iyakevdgkl iecspdeatt tkfsvkcagm tdtikkkvtf
541 dnfrvgfsst gkpkpvqvng gvvldsvft ik

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[00520] (SEQ ID NO: 33)

[00521] In some embodiments, the polymerase of the labeled polymerase conjugate comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 33, or any biologically active fragment thereof.

[00522] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes one or more mutations reducing the 3' to 5' exonuclease activity of the polymerase. In some embodiments, the one or more mutations reducing the 3' to 5' exonuclease activity are selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G.

[00523] In some embodiments, the polymerase of the labeled polymerase conjugate comprises the amino acid of SEQ ID NO: 34, below:

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1 mprkmfscdf etttklddcr vwaygyeig nldnykigns ldefmqwvme iqadlyfhnl
61 kfdgafivnw lehghfkwsn eglpntynti iskmqgwymi dicfgykgkr klhtviyds1
121 kklpfpvkki akdfqlpllk gdidyhaerp vgheitpeey eyiknaieii araldiqfkq
181 glrmtagsd slkgfkdlis tkkfnkvfpk lslpmdkeir rayrggftwl ndkyekeig
241 egmvfdvns1 ypsqmysrpl pygapivfqg kyekdeqypl yiqrirfefe lkegyiptiq
301 ikknppffkgn eylknsgaep velyltnvdl eliqehyemy nveyidgfkf rektglfkef
361 idkwtyvkth ekgakkqlak lmlnslygkf asnpdvtgkv pylkedgslg frvgdeeykd
421 pvytpmgvfi tawarfttit aagacydrii ycdtdsihlt gtevpeiikd ivdpkklgyw
481 ahestfkrak ylrqktyiqd iyakevdgkl iecspdeatt tkfsvkcagm tdtikkkvtf
541 dnfrvgfsst gkpkpvqvng gvvldsvft ik

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(SEQ ID NO: 34)

[00524] In some embodiments, the polymerase of the labeled polymerase conjugate comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 34, or any biologically active fragment thereof. Typically, the polymerase of SEQ ID NO: 34 will exhibit reduced exonuclease activity relative to a reference polymerase comprising the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 32 or SEQ ID NO: 33.

[00525] In some embodiments, the polymerase of the labeled polymerase conjugate is a variant of a B103 polymerase comprising the amino acid sequence of SEQ ID NO: 32, SEQ ID NO: 33 or SEQ ID NO: 34, wherein the variant further comprises one, two, three or more modifications at amino acid positions 2, 9, 58, 59, 63, 129, 166, 246, 247, 339, 370, 371, 372, 373, 374, 375, 376, 377, 380, 383, 384, 385, 455, 507 and 509, or any combinations thereof, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00526] In some embodiments, the amino acid sequence of the polymerase of the labeled polymerase conjugate is fused to a peptide sequence that encodes a stretch of amino acid acids capable of functioning as a peptide linker to facilitate the formation of a linkage between the polymerase and another reactive moiety. The reactive moiety can in some embodiments be a label, or another attachment moiety that is itself linked to one or more labels. This peptide linker sequence can be fused to the N-terminus, the C-terminus or any suitable position between the N-terminus and the C-terminus of the polymerase.

[00527] In some embodiments, the polymerase is derived from a Phi-29-like polymerase and comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes amino acid mutations at any one, two, three or more positions selected from the group consisting of: 2, 9, 12, 14, 15, 58, 59, 61, 63, 73, 98, 107, 129, 147, 166, 176, 185, 186, 187, 195, 208, 221, 246, 247, 248, 251, 252, 256, 300, 302, 310, 318, 339, 357, 359, 360, 362, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 380, 383, 384, 385, 386, 387, 389, 390, 392, 399, 405, 411, 419, 430, 455, 475, 477, 481, 483, 493, 494, 497, 503, 507, 509, 511, 526, 528, 529, 531, 535, 544, 550, 552, 555, 567, 569 and 572, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the modifications can include deletions, additions and substitutions. The substitutions can be conservative or non-conservative substitutions. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes one or more mutations reducing the 3' to 5' exonuclease activity of the polymerase. In some embodiments, the one or more mutations reducing the 3' to 5' exonuclease activity are selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G.

[00528] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes any one, two, three or more amino acid mutations selected from the group consisting of: T365G, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W, T365Y, T365H, H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y, H370F, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, F383L, D384N, A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W, A481Y, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the

numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, this polymerase comprises the amino acid substitution H370R and/or K380R.

[00529] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes an amino acid mutation selected from the group: H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y and H370F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increased t_{-1} value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 33. In some embodiments, the t_{-1} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the t_{-1} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00530] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes an amino acid mutation selected from the group: K380G, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y and K380F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increased t_{pol}

value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 33. In some embodiments, the t_{pol} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes the amino acid mutation K380R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00531] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 and further includes an amino acid mutation selected from the group: T373G, T373E, T373T, T373S, T373R, T373A, K T373Q, T373W, T373Y and T373F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3. Typically, this polymerase can exhibit an increased t_{11} value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 3. In some embodiments, the t_{11} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the t_{11} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D12A, E14I, E14A, T15I, N62D, D66A, Y165F, Y165C, and D169A, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 and further includes the amino acid mutation T373R,

wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the polymerase further includes the mutations D12A and D66A.

[00532] In some embodiments, the biomolecule is derived from a M2 polymerase (also known as M2Y DNA polymerase) having the amino acid sequence of the SEQ ID NO: 35 as follows:

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1   msrkmfscdf etttklddcr vwaygymeig nldnykigns ldefmqwvme iqadlyfhnl
61  kfdgafivnw leqhgfkwsn eglpntynti iskmqgwyim dicfgykgkr klhtviyds1
121 kklpfpvkki akdfqlpllk gdidyhterp vgheitpeey eyikndieii araldiqfkq
181 gldrmtagsd slkgfkdlis tkkfinkvfpk lslpmdkeir kayrggftwl ndkykekeig
241 egmvfdvnsl ypsqmysrpl pygapivfqq kyekdeqypl yiqrirfefe lkegyiptiq
301 ikknppffkgn eylknsgevpe velyltnvdl eliqehyely nveyidgfkf rektglfkdf
361 idkwtyvkth eegakkqlak lmlnslygkf asnpdvtgkv pylkddgslg frvgdeeykd
421 pvytpmgvfi tawarfttit aagacydrii ycdtdsihlt gtevpeiikd ivdpkklgyw
481 ahestfkrak ylrqktyiqd iyvkevdgkl kecspeatt tkfsvkcagm tdtikkkvtf
541 dnfavgfssm gkpkpvqvng gvvldsvft ik

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(SEQ ID NO: 35)

[00533] In some embodiments, the polymerase comprises an amino acid sequence that is at least 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 35 and further comprises an amino acid mutation at one, two, three or more amino acid positions selected from the group consisting of: 9, 11, 12, 58, 59, 63, 162, 162, 166, 377 and 385, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, such a polymerase will exhibit reduced 3' to 5' exonuclease activity relative to reference polymerase having the amino acid sequence of SEQ ID NO: 35.

[00534] In some embodiments, the polymerase comprises an amino acid sequence that is at least 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 35 and further comprises one, two, three or more amino acid mutations selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises any one, two, three, four, five or all of these mutations. In some embodiments, the polymerase comprises the amino acid substitution D166A. In some embodiments, the polymerase comprises the amino acid substitutions D9A and D63A. In some embodiments, the polymerase comprises the amino acid substitutions N59D and T12I. Typically, such polymerases will exhibit reduced 3' to 5'

exonuclease activity relative to reference polymerase having the amino acid sequence of SEQ ID NO: 35.

[00535] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further comprises an amino acid substitution at one or more positions selected from the group consisting of: 2, 73, 147, 221, 318, 339, 359, 372, 405, 503, 511, 544 and 550, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00536] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00537] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes a mutation at position 370. In some embodiments, the mutation is selected from the group consisting of: H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y and H370F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation H370R. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00538] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes a mutation at position 365, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: T365H, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W and T365Y, wherein

the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation T365F. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00539] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes a mutation at position 372, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y and K372F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation K372Y. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00540] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes a mutation at position 481, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W and A481Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation A481E. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00541] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes a mutation at position 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: K509E, K509F, K509G, K509S, K509R, K509K, K509A, K509T, K509Q, K509W and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation K509Y. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00542] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes an amino acid mutation at any one, two, three or more positions selected from the group consisting of: 9, 12, 14, 15, 58, 59, 61, 63, 98, 129, 176, 185, 186, 187, 195, 208, 246, 247, 248, 251, 252, 256, 300, 302, 310, 357, 360, 362, 365, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 380, 383, 384, 385, 386, 387, 389, 390, 392, 399, 411, 419, 430, 455, 475, 477, 481, 483, 493, 494, 497, 507, 509, 511, 526, 528, 529, 531, 535, 544, 555, 567, 569 and 572, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the modifications can include deletions, additions and substitutions. The substitutions can be conservative or non-conservative substitutions. Optionally, this polymerase comprises the amino acid substitution H370R. In some embodiments, the polymerase further comprises one or more mutations reducing the exonuclease activity as described herein such as, for example, the amino acid substitution D166A. Typically, this polymerase can exhibit increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00543] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further comprise amino acid mutations at any one, two, three or more positions selected from the group consisting of: 2, 9, 12, 14, 15, 58, 59, 61, 63, 73, 98, 129, 147, 166, 176, 185, 186, 187, 195, 208, 221, 246, 247, 248, 251, 252, 256, 300, 302, 310, 318, 339, 357, 359, 360, 362, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 380, 383, 384, 385, 386, 387, 389, 390, 392, 399, 405, 411, 419, 430, 455, 475, 477, 481, 483, 493, 494, 497, 503, 507, 509, 511, 526, 528, 529, 531, 535, 544, 550, 552, 555, 567, 569 and 572, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the modifications can include deletions, additions and substitutions. The substitutions can be conservative or non-conservative substitutions. Optionally, this polymerase comprises the amino acid substitution H370R. In some embodiments, the polymerase further comprises one or more mutations reducing the exonuclease activity as described herein such as, for example, the amino acid substitution D166A. Typically, this polymerase can exhibit increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00544] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes any one, two, three or more amino acid mutations selected from the group consisting of: T365G, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W, T365Y, T365H, H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y, H370F, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W, A481Y, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A,

K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, this polymerase can exhibit an increased branching ratio and/or increased t_{-1} value relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or t_{-1} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the branching ratio and/or t_{-1} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the branching ratio and/or t_{-1} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00545] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes amino acid mutations at positions 372 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes the amino acid substitutions E372Y and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes amino acid mutations at positions 365, 372 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes the amino acid substitutions T365F, E372Y and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%,

85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes amino acid mutations at positions 365, 372, 481 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes the amino acid substitutions T365F, E372Y, A481E and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, such polymerases can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00546] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further comprises the amino acid mutation H370R. Optionally, the polymerase can further comprise any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, R107K, T147A, K221R, V318A, L339M, D359E, E372K, D405E, V503A, K511I, A544R, M550T, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, this polymerase can exhibit increased branching ratio and/or increased t_{-1} value and/or t_{-1} increased t_{pol} value relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the branching ratio and/or t_{-1} value and/or

t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00547] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further comprises any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, the polymerase can further comprise one, two or three amino acid mutations selected from the group: T365G, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W, T365Y, T365H, H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y, H370F, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W, A481Y, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, this polymerase can exhibit increase branching ratio in the presence of the dye-labeled nucleotide AF647-C6-dG6P relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R,

N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00548] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes an amino acid mutation selected from the group: H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y and H370F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increase t_{-1} value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the t_{-1} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, the polymerase can further include any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the t_{-1} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00549] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 35 and further includes an amino acid mutation selected from the group: K380G, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y and K380F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increased t_{pol}

value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 35. In some embodiments, the t_{pol} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. In some embodiments, the t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, the polymerase can further include any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes the amino acid mutation K380R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00550] In some embodiments, the polymerase is derived from a bacteriophage Nf polymerase having the amino acid sequence of the SEQ ID NO: 36 as follows:

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1  msrkmfscdf etttklddcr vwaygyeig nldnykigns ldefmqwvme iqadlyfhnl
61  kfdgafivnw leqhgfkwsn eglpntynti iskmqgwymi dicfgyrgkr klhtviyds1
121 kklpfpvkki akdfqlpllk gdidyhterp vgheitpeey eyikndieii araldiqfkq
181 gldrmtagsd slkgfkdlis tkkfinkvfpk lslpmdkeir kayrggftwl ndkykekeig
241 egmvfdvns1 ypsqmysrpl pygapivfqg kyekdeqypl yiqrirfefe lkegyiptiq
301 ikknppffkgn eylknsgvep velyltnvdl eliqehyely nveyidgfkf rektglfkdf
361 idkwtyvkth eegakkqlak lmlnslygkf asnpdvtgkv pylkddgslg frvgdeeykd
421 pvytpmgvfi tawarfttit aagacydrii ycdtdsihlt gtevpeiikd ivdpkklgyw
481 ahestfkrak ylrqktyiqd iyvkevdgkl kecspdeatt tkfsvkcagm tdtikkkvtf
541 dnfavgfssm gkpkpvqvng gvvldsvft ik

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(SEQ ID NO: 36)

[00551] In some embodiments, the polymerase can comprise an amino acid sequence that is at least 85%, 90%, 95%, 97%, 98% or 99% or 100% identical to the amino acid of SEQ ID NO: 36.

[00552] In some embodiments, the polymerase comprises an amino acid sequence that is at least 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid

sequence of SEQ ID NO: 36 and further comprises an amino acid mutation at one, two, three or more amino acid positions selected from the group consisting of: 9, 11, 12, 58, 59, 63, 162, 162, 166, 377 and 385, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, such a polymerase will exhibit reduced 3' to 5' exonuclease activity relative to reference polymerase having the amino acid sequence of SEQ ID NO: 36.

[00553] In some embodiments, the polymerase comprises an amino acid sequence that is at least 85%, 90%, 95%, 97%, 98% or 99% identical to the amino acid sequence of SEQ ID NO: 36 and further comprises one, two, three or more amino acid mutations selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises any one, two, three, four, five or all of these mutations. In some embodiments, the polymerase comprises the amino acid substitution D166A. In some embodiments, the polymerase comprises the amino acid substitutions D9A and D63A. In some embodiments, the polymerase comprises the amino acid substitutions N59D and T12I. Typically, such polymerases will exhibit reduced 3' to 5' exonuclease activity relative to reference polymerase having the amino acid sequence of SEQ ID NO: 36.

[00554] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further comprises an amino acid substitution at one or more positions selected from the group consisting of: 2, 73, 107, 147, 221, 318, 339, 359, 372, 405, 503, 511, 544 and 550, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00555] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes a mutation at position 370. In some embodiments, the mutation is selected from the group consisting of: H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y and H370F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation H370R. Typically,

this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00556] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes a mutation at position 365, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: T365H, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W and T365Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation T365F. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00557] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes a mutation at position 372, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y and K372F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation K372Y. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00558] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes a mutation at position 481, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In

some embodiments, the mutation is selected from the group consisting of: A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W and A481Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation A481E. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00559] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes a mutation at position 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the mutation is selected from the group consisting of: K509E, K509F, K509G, K509S, K509R, K509K, K509A, K509T, K509Q, K509W and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, this polymerase comprises the amino acid mutation K509Y. Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00560] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, R107K, T147A, K221R, V318A, L339M, D359E, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00561] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes an amino acid mutation

at any one, two, three or more positions selected from the group consisting of: 9, 12, 14, 15, 58, 59, 61, 63, 98, 129, 176, 185, 186, 187, 195, 208, 246, 247, 248, 251, 252, 256, 300, 302, 310, 357, 360, 362, 365, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 380, 383, 384, 385, 386, 387, 389, 390, 392, 399, 411, 419, 430, 455, 475, 477, 481, 483, 493, 494, 497, 507, 509, 511, 526, 528, 529, 531, 535, 544, 555, 567, 569 and 572, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the modifications can include deletions, additions and substitutions. The substitutions can be conservative or non-conservative substitutions. Optionally, this polymerase comprises the amino acid substitution H370R. In some embodiments, the polymerase further comprises one or more mutations reducing the exonuclease activity as described herein such as, for example, the amino acid substitution D166A. Typically, this polymerase can exhibit increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00562] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further comprise amino acid mutations at any one, two, three or more positions selected from the group consisting of: 2, 9, 12, 14, 15, 58, 59, 61, 63, 73, 98, 107, 129, 147, 166, 176, 185, 186, 187, 195, 208, 221, 246, 247, 248, 251, 252, 256, 300, 302, 310, 318, 339, 357, 359, 360, 362, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 380, 383, 384, 385, 386, 387, 389, 390, 392, 399, 405, 411, 419, 430, 455, 475, 477, 481, 483, 493, 494, 497, 503, 507, 509, 511, 526, 528, 529, 531, 535, 544, 550, 552, 555, 567, 569 and 572, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the modifications can include deletions, additions and substitutions. The substitutions can be conservative or non-conservative substitutions. Optionally, this polymerase comprises the amino acid substitution H370R. In some embodiments, the polymerase further comprises one or more mutations reducing the exonuclease activity as described herein such as, for example, the amino acid substitution D166A. Typically, this polymerase can exhibit increased branching ratio and/or increased

nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00563] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes any one, two, three or more amino acid mutations selected from the group consisting of: T365G, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W, T365Y, T365H, H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y, H370F, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W, A481Y, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises the amino acid mutations Typically, this polymerase can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00564] In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes amino acid mutations at positions 372 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid

sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes the amino acid substitutions E372Y and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes amino acid mutations at positions 365, 372 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes the amino acid substitutions T365F, E372Y and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes amino acid mutations at positions 365, 372, 481 and 509, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes the amino acid substitutions T365F, E372Y, A481E and K509Y, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, such polymerases can exhibit an increased branching ratio and/or increased nucleotide binding affinity relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio and/or nucleotide binding affinity is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32.

[00565] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further comprises the amino acid

mutation H370R. Optionally, the polymerase can further comprise any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, R107K, T147A, K221R, V318A, L339M, D359E, E372K, D405E, V503A, K511I, A544R, M550T, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, D507H, D507G, D507E, D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, this polymerase can exhibit increased branching ratio, increased t_1 and/or increased t_{pol} values relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the branching ratio, t_1 value and/or t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00566] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further comprises any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Optionally, the polymerase can further comprise one, two or three amino acid mutations selected from the group: T365G, T365F, T365G, T365S, T365K, T365R, T365A, T365Q, T365W, T365Y, T365H, H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y, H370F, E371G, E371H, E371T, E371S, E371K, E371R, E371A, E371Q, E371W, E371Y, E371F, K372G, K372E, K372T, K372S, K372R, K372A, K372Q, K372W, K372Y, K372F, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y, K380F, A481E, A481F, A481G, A481S, A481R, A481K, A481A, A481T, A481Q, A481W, A481Y, D507H, D507G, D507E,

D507T, D507S, D507R, D507A, D507R, D507Q, D507W, D507Y, D507F, K509H, K509G, K509D, K509R, K509E, K509T, K509S, K509R, K509A, K509Q, K509W, K509Y and K509F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. Typically, this polymerase can exhibit increase branching ratio and/or increased t_{11} value and/or increased t_{pol} value relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the branching ratio, t_{11} value and/or t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P.

[00567] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes an amino acid mutation selected from the group: H370G, H370T, H370S, H370K, H370R, H370A, H370Q, H370W, H370Y and H370F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increase t_{11} value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the t_{11} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, the polymerase can further include any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the t_{11} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. In some embodiments, the polymerase comprises an amino acid

sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes the amino acid mutation H370R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00568] In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 36 and further includes an amino acid mutation selected from the group: K380G, K380E, K380T, K380S, K380R, K380A, K380Q, K380W, K380Y and K380F, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Typically, this polymerase can exhibit an increased t_{pol} value in the presence of the dye-labeled nucleotides relative to a reference polymerase having the amino acid sequence of SEQ ID NO: 36. In some embodiments, the t_{pol} value of the polymerase is increased by at least about 105%, 110%, 125%, 150%, 175%, 200%, 250%, 500%, 750%, or 1000% relative to the reference polymerase. Optionally, the polymerase can further include one or more mutations reducing 3' to 5' exonuclease activity selected from the group consisting of: D9A, E11A, E11I, T12I, H58R, N59D, D63A, Y162F, Y162C, D166A, Q377A and S385G, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33. Optionally, the polymerase can further include any one, two, three or more amino acid mutations selected from the group consisting of: S2P, Q73H, T147A, K221R, V318A, L339M, D359E, H370R, E372K, D405E, V503A, K511I, A544R and M550T, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 32. In some embodiments, the t_{pol} value is increased in the presence of the dye-labeled nucleotide AF647-C6-dG6P. In some embodiments, the polymerase comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 33 and further includes the amino acid mutation K380R, wherein the numbering is relative to the amino acid sequence of SEQ ID NO: 33.

[00569] In some embodiments, the biomolecule is a Phi-29 polymerase comprising the TEV protease recognition sequence at its N-terminal end. Optionally, the biomolecule can also comprise a His tag. The His-tag can be fused to the N-terminus, the C-terminus or any other suitable position of the Phi-29 polymerase. Optionally,

the His-tag can be separated from the amino acid residues of the protein by a linker comprising the TEV protease recognition sequence. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MNHKVVHHHH	HIEGRENLYF	QCMELGTLG	SMKHMPRKMY	SCAFETTTKV	EDCRVWAYGY
70	80	90	100	110	120
MNIEDHSEYK	IGNSLDEFMA	WVLKVQADLY	FHNLFKAGAF	IINWLERNGF	KWSADGLPNT
130	140	150	160	170	180
YNTIISRMGQ	WYMIDICLGY	KGKRKIHTVI	YDSLKKLPFP	VKKIAKDFKL	TVLKGDIDYH
190	200	210	220	230	240
KERPVGKIT	PEEYAYIKND	IQIIAEALLI	QFKQGLDRMT	AGSDSLKGFK	DIITTKKFKK
250	260	270	280	290	300
VFPTLSLGLD	KEVRYAYRGG	FTWLNDRFKE	KEIGEGMVFD	VNSLYPAQMY	SRLLPYGEP
310	320	330	340	350	360
VFEGKYVWDE	DYPLHIQHIR	CEFELKEGYI	PTIIQIKSRF	YKGNEYLKSS	GGEIADLWLS
370	380	390	400	410	420
NVDLELMKEH	YDLYNVEYIS	GLKFKATTGL	FKDFIDKWTY	IKTTSEGAIK	QLAKLMLNSL
430	440	450	460	470	480
YGKFASNPDV	TGKVPYLKEN	GALGFRLGEE	ETKDPVYTPM	GVFITAWARY	TTITAAQACY
490	500	510	520	530	540
DRIIYCDTDS	IHLTGTEIPD	VIKDIVDPKK	LGYWAHESTF	KRAKYLRQKT	YIQDIYMKEV
550	560	570	580	590	600
DGKLVEGSPD	DYTDIKFSVK	CAGMTDKIKK	EVTFFENFKVG	FSRKMKPKPV	QVPGGVVLVD

DTFTIK

(SEQ ID NO: 38)

[00570] In some embodiments, the biomolecule is a mutant Phi-29-like polymerase comprising the TEV protease recognition sequence at its N-terminal end. Optionally, the biomolecule can also comprise a His tag. The His-tag can be fused to the N-terminus, the C-terminus or any other suitable position of the mutant B103 polymerase. Optionally, the His-tag can be separated from the amino acid residues of the protein by a linker comprising the TEV protease recognition sequence. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MHHHHHLLG	GGGENLYFQC	GGGGSAAAGS	AARKMFSCDF	ETTTKLDDCR	VWAYGYMEIG
70	80	90	100	110	120
NLDNYKIGNS	LDEFMQWVME	IQADLYFHNL	KFDGAFIVNW	LEHHGFKWSN	EGLPNTYNTI
130	140	150	160	170	180
ISKMGQWYMI	DICFGYKGKR	KLHTVIYDSL	KKLPFPVKKI	AKDFQLPLLK	GDIDYHAERP
190	200	210	220	230	240
VGHEITPEEY	EYIKNAIEII	ARALDIQFKQ	GLDRMTAGSD	SLKGFKDILS	TKKFNVKVPK
250	260	270	280	290	300
LSLPMDKEIR	RAYRGGFTWL	NDKYKEKEIG	EGMVFDVNSL	YPSQMYSRPL	PYGAPIVFQG
310	320	330	340	350	360
KYEKDEQYPL	YIQRIRFEFE	LKEGYIPTIQ	IKKNPFCKGN	EYLNKSGAEP	VELYLTNVDL
370	380	390	400	410	420
ELIQEHYEMY	NVEYIDGFKF	REKTGLFKEF	IDKWTYVKTH	EKGAKQLAK	LMLNSLYGKF
430	440	450	460	470	480
ASNPDVTGKV	PYLKEDGSLG	FRVGDEEYKD	PVYTPMGVFI	TAWARFTTIT	AAQACYDRII
490	500	510	520	530	540
YCDTDSIHLT	GTEVPEIIKD	IVDPKKLGyw	AHESTFKRAK	YLRQKTYIQD	IYAKEVDGKL
550	560	570	580	590	600
IECSPDEATT	TKFSVKCAGM	TDTIKKKVTF	DNFRVGFSS	GKPKPVQVNG	GVVLVDSVFT

(SEQ ID NO: 39)

[00571] In some embodiments, the biomolecule is a fusion protein comprising a mutant Phi-29-like polymerase comprising a His tag linked to its N-terminal end, and including one or more amino acid substitutions selected from the group consisting of D166A, H370R, F383L and D384N, wherein the numbering is relative to the amino acid sequence of wild type B103 having the amino acid sequence of SEQ ID NO: 32. In some embodiments, the fusion protein comprises all four amino acid substitutions. In some embodiments, the fusion protein comprises an amino acid sequence that is at least 70%, 80%, 85%, 90%, 95%, 99% or 100% identical to the following amino acid sequence:

10	20	30	40	50	60
MSHHHHHSM	SGLNDIFEAQ	KIEWHEGAPG	ARGSKHMPRK	MFSCDFETTT	KLDDCRVWAY
70	80	90	100	110	120
GYMEIGNLDN	YKIGNSLDEF	MQWVMEIQAD	LYFHNLFKFDG	AFIVNWLEHH	GFKWSNEGLP
130	140	150	160	170	180
NTYNTIISKM	GQWYMIDICF	GYKGKRLHT	VIYDSLKKLP	FPVKKIADKF	QLPLLKGDID
190	200	210	220	230	240
YHAERPVGHE	ITPEEYIYIK	NAIEIIARAL	DIQFKQGLDR	MTAGSDSLKG	FKDILSTKKF
250	260	270	280	290	300
NKVFPKLSLP	MDKEIRRAYR	GGFTWLNDKY	KEKEIGEGMV	FDVNSLYPSQ	MYSRPLPYGA
310	320	330	340	350	360
PIVFQGYEK	DEQYPLYIQR	IRFEFELKEG	YIPTIQIKKN	PFFKGNEYLK	NSGAEPVELY
370	380	390	400	410	420
LTVNDELTLQ	EHYEMYNVEY	IDGFKFREKT	GLFKEFIDKW	TYVKTRKGA	KKQLAKMLN
430	440	450	460	470	480

SLYGKFASNP DVTGKVPYLK EDGSLGFRVG DEEYKDPVYT PMGVFITAWA RFTTITAAQA
 490 500 510 520 530 540
 CYDRIIYCDT DSIHLTGTEV PEIIKDIVDP KKLGYWAHES TFKRAKYLRQ KTYIQDIYAK
 550 560 570 580 590 600
 EVDGKLI ECS PDEATTTKFS VKCAGMTDTI KKKVTFDNFR VGFSSTGKPK PVQVNGGVVL
 VDSVFIIK

(SEQ ID NO: 40)

[00572] As the skilled artisan will readily appreciate, the scope of the present disclosure encompasses not only the specific amino acid and/or nucleotide sequences disclosed herein, but also, for example, to many related sequences encoding genes and/or peptides with the functional properties described herein. For example, the nucleotide and amino acid sequence of the polymerase of the labeled polymerase conjugate can include any nucleotide and amino acid sequence encoding conservative variants of the polymerases disclosed herein are also within the scope of the present disclosure.

[00573] In some embodiments, the methods, compositions, systems and/or kits disclosed herein can involve the use of moieties capable of undergoing energy transfer. Such energy transfer moieties can include energy transfer donors and acceptors. The energy transfer moieties can be linked to the solid surfaces, nanoparticles, polymerases, nucleotides, target nucleic acid molecules, primers, and/or oligonucleotides.

[00574] In one exemplary embodiment, the labeled biomolecule conjugates of the present disclosure comprise a polymerase linked to a label that includes an energy transfer moiety, wherein the conjugate has polymerase activity. In some embodiments, the energy transfer moiety of the conjugate performs energy transfer, which can be RET or FRET. In some embodiments, the energy transfer moiety of the conjugate performs energy transfer with the label of a nucleotide.

[00575] In one aspect, the energy transfer moiety can be an energy transfer donor. For example, the energy transfer donor can be a nanoparticle or an energy transfer donor moiety (e.g., fluorescent dye). In another aspect, the energy transfer moiety can be an energy transfer acceptor. For example, the energy transfer acceptor can be an energy acceptor dye. In another aspect, the energy transfer moiety can be a quencher moiety.

[00576] In one aspect, the energy transfer pair can be linked to the same molecule. For example, the energy transfer donor and acceptor pair can be linked to a single polymerase, which can provide detection of conformational changes in the polymerase. In another aspect, the donor and acceptor can be linked to different molecules in any combination. For example, the donor can be linked to the polymerase, target molecule, or primer molecule, and/or the acceptor can be linked to the nucleotide, the target molecule, or the primer molecule.

[00577] The energy transfer donor is capable of absorbing electromagnetic energy (e.g., light) at a first wavelength and emitting excitation energy in response. The energy acceptor is capable of absorbing excitation energy emitted by the donor and fluorescing at a second wavelength in response.

[00578] The donor and acceptor moieties can interact with each other physically or optically in a manner which produces a detectable signal when the two moieties are in proximity with each other. A proximity event includes two different moieties (e.g., energy transfer donor and acceptor) approaching each other, or associating with each other, or binding each other.

[00579] The donor and acceptor moieties can transfer energy in various modes, including: fluorescence resonance energy transfer (FRET) (L. Stryer 1978 *Ann. Rev. Biochem.* 47: 819-846; Schneider, U.S. Patent No. 6,982,146; Hardin, U.S. Patent No. 7,329,492; Hanzel U.S. published patent application No. 2007/0196846), scintillation proximity assays (SPA) (Hart and Greenwald 1979 *Molecular Immunology* 16:265-267; U.S. Pat. No. 4,658,649), luminescence resonance energy transfer (LRET) (G. Mathis 1995 *Clin. Chem.* 41:1391-1397), direct quenching (Tyagi et al, 1998 *Nature Biotechnology* 16:49-53), chemiluminescence energy transfer (CRET) (Campbell and Patel 1983 *Biochem. Journal* 216:185-194), bioluminescence resonance energy transfer (BRET) (Y. Xu, et al., 1999 *Proc. Natl. Acad. Sci.* 96:151-156), and excimer formation (J. R. Lakowicz 1999 "Principles of Fluorescence Spectroscopy", Kluwer Academic/Plenum Press, New York).

[00580] In one exemplary embodiment, the energy transfer moieties can be a FRET donor/acceptor pair. FRET is a distance-dependent radiationless transmission of excitation energy from a first moiety, referred to as a donor moiety, to a second moiety, referred to as an acceptor moiety. Typically, the efficiency of FRET energy

transmission is dependent on the inverse sixth-power of the separation distance between the donor and acceptor, r . For a typical donor-acceptor pair, r can vary between approximately 10-100 Angstroms. FRET is useful for investigating changes in proximity between and/or within biological molecules. In some embodiments, FRET efficiency may depend on donor-acceptor distance r as $1/r^6$ or $1/r^4$. The efficiency of FRET energy transfer can sometimes be dependent on energy transfer from a point to a plane which varies by the fourth power of distance separation (E. Jares-Erijman, et al., 2003 Nat. Biotechnol. 21:1387). The distance where FRET efficiency is at 50% is termed R_0 , also known as the Forster distance. R_0 can be unique for each donor-acceptor combination and can range from between about 5nm to about 10nm. A change in fluorescence from a donor or acceptor during a FRET event (e.g., increase or decrease in the signal) can be an indication of proximity between the donor and acceptor.

[00581] FRET efficiency (E) can be defined as the quantum yield of the energy transfer transition, *i.e.* the fraction of energy transfer event occurring per donor excitation event. It is a direct measure of the fraction of photon energy absorbed by the donor which is transferred to an acceptor, as expressed in Equation 1: $E = k_{ET} / k_f + k_{ET} + \sum k_i$ where k_{ET} is the rate of energy transfer, k_f the radiative decay rate and the k_i are the rate constants of any other de-excitation pathway.

[00582] FRET efficiency E generally depends on the inverse of the sixth power of the distance r (nm) between the two fluorophores (*i.e.*, donor and acceptor pair), as expressed in Equation 2: $E = 1 / 1 + (r/R_0)^6$.

[00583] Therefore, the FRET efficiency of a donor describes the maximum theoretical fraction of photon energy which is absorbed by the donor (*i.e.*, nanoparticle) and which can then be transferred to a typical organic dye (e.g., fluoresceins, rhodamines, cyanines, etc.).

[00584] In biological applications, FRET can provide an on-off type signal indicating when the donor and acceptor moieties are proximal (e.g., within R_0) of each other. Additional factors affecting FRET efficiency include the quantum yield of the donor, the extinction coefficient of the acceptor, and the degree of spectral overlap between the donor and acceptor. Procedures are well known for maximizing the FRET signal and detection by selecting high yielding donors and high absorbing acceptors with the

greatest possible spectral overlap between the two (D. W. Piston and G.J. Kremers 2007 Trends Biochem. Sci. 32:407). Resonance energy transfer may be either an intermolecular or intramolecular event. Thus, the spectral properties of the energy transfer pair as a whole, change in some measurable way if the distance and/or orientation between the moieties are altered.

[00585] The production of signals from FRET donors and acceptors can be sensitive to the distance between donor and acceptor moieties, the orientation of the donor and acceptor moieties, and/or a change in the environment of one of the moieties (Deuschle *et al.* 2005 Protein Science 14: 2304-2314; Smith *et al.* 2005 Protein Science 14:64-73). For example, a nucleotide linked with a FRET moiety (e.g., acceptor) may produce a detectable signal when it approaches, associates with, or binds a polymerase linked to a FRET moiety (e.g., donor). In another example, a FRET donor and acceptor linked to one protein can emit a FRET signal upon conformational change of the protein. Some FRET donor/acceptor pairs exhibit changes in absorbance or emission in response to changes in their environment, such as changes in pH, ionic strength, ionic type (NO_2^- , Ca^{+2} , Mg^{+2} , Zn^{+2} , Na^+ , Cl^- , K^+), oxygen saturation, and solvation polarity.

[00586] The FRET donor and/or acceptor may be a fluorophore, luminophore, chemiluminophore, bioluminophore, or quencher (P. Selvin 1995 Methods Enzymol 246:300-334; C. G. dos Remedios 1995 J. Struct. Biol. 115:175-185; P. Wu and L. Brand 1994 Anal Biochem 218:1-13).

[00587] In some embodiments, the energy transfer moieties may not undergo FRET, but may undergo other types of energy transfer with each other, including luminescence resonance energy transfer, bioluminescence resonance energy transfer, chemiluminescence resonance energy transfer, and similar types of energy transfer not strictly following the Forster's theory, such as the non-overlapping energy transfer when non-overlapping acceptors are utilized (Laitala and Hemmila 2005 Anal. Chem. 77: 1483-1487).

[00588] In one embodiment, the polymerase can be linked to an energy transfer donor moiety. In another embodiment, the nucleotide can be linked to an energy transfer acceptor moiety. For example, in one embodiment the nucleotide comprises a polyphosphate chain and an energy transfer moiety linked to the the terminal

phosphate group of the polyphosphate chain. A change in a fluorescent signal can occur when the labeled nucleotide is proximal to the labeled polymerase.

[00589] In one embodiment, when an acceptor-labeled nucleotide is proximal to a donor-labeled polymerase, the signal emitted by the donor moiety decreases. In another embodiment, when the acceptor-labeled nucleotide is proximal to the donor-labeled polymerase, the signal emitted by the acceptor moiety increases. In another embodiment, a decrease in donor signal and increase in acceptor signal correlates with nucleotide binding to the polymerase and/or correlates with polymerase-dependent nucleotide incorporation.

[00590] Quenchers

[00591] The energy transfer moiety can be a FRET quencher. Typically, quenchers have an absorption spectrum with large extinction coefficients, however the quantum yield for quenchers is reduced, such that the quencher emits little to no light upon excitation. Quenching can be used to reduce the background fluorescence, thereby enhancing the signal-to-noise ratio. In one aspect, energy transferred from the donor may be absorbed by the quencher which emits moderated (e.g., reduced) fluorescence. In another aspect, the acceptor can be a non-fluorescent chromophore which absorbs the energy transferred from the donor and emits heat (e.g., the energy acceptor is a dark quencher).

[00592] For an example, a quencher can be used as an energy acceptor with a nanoparticle donor in a FRET system, see I.L. Medintz, et al., 2003 Nature Materials 2:630. One exemplary method involves the use of quenchers in conjunction with reporters comprising fluorescent reporter moieties. In this strategy, certain nucleotides in the reaction mixture are labeled with a reporter comprising a fluorescent label, while the remaining nucleotides are labeled with a quencher. Alternatively, each of the nucleotides in the reaction mixture is labeled with a quencher. Discrimination of the nucleotide bases is based on the wavelength and/or intensity of light emitted from the FRET acceptor, as well as the intensity of light emitted from the FRET donor. If no signal is detected from the FRET acceptor, a corresponding reduction in light emission from the FRET donor indicates

incorporation of a nucleotide labeled with a quencher. The degree of intensity reduction may be used to distinguish between different quenchers.

[00593] Examples of fluorescent donors and non-fluorescent acceptor (e.g., quencher) combinations have been developed for detection of proteolysis (Matayoshi 1990 Science 247:954-958) and nucleic acid hybridization (L. Morrison, in: Nonisotopic DNA Probe Techniques, ed., L. Kricka, Academic Press, San Diego, (1992) pp. 31 1-352; S. Tyagi 1998 Nat. Biotechnol. 16:49-53; S. Tyagi 1996 Nat. Biotechnol. 14:947-8). FRET donors, acceptors and quenchers can be moieties which absorb electromagnetic energy (e.g., light) at about 300-900 nm, or about 350-800 nm, or about 390-800 nm.

[00594] Materials for Energy Transfer Moieties

[00595] Energy transfer donor and acceptor moieties can be made from materials which typically fall into four general categories (see the review in: K. E. Sapford, et al., 2006 Angew. Chem. Int. Ed. 45:4562-4588), including: (1) organic fluorescent dyes, dark quenchers and polymers (e.g., dendrimers); (2) inorganic material such as metals, metal chelates and semiconductor nanoparticles; (3) biomolecules such as proteins and amino acids (e.g., green fluorescent protein and derivatives thereof); and (4) enzymatically catalyzed bioluminescent molecules. The material for making the energy transfer donor and acceptor moieties can be selected from the same or different categories.

[00596] The FRET donor and acceptor moieties which are organic fluorescent dyes, quenchers or polymers can include include traditional dyes which emit in the UV, visible, or near-infrared region. The UV emitting dyes include coumarin-, pyrene-, and naphthalene-related compounds. The visible and near-infrared dyes include xanthene-, fluorescein-, rhodol-, rhodamine-, and cyanine-related compounds. The fluorescent dyes also includes DDAO ((7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)), resorufin, ALEXA FLUOR and BODIPY dyes (both Molecular Probes), HILYTE Fluors (AnaSpec), ATTO dyes (Atto-Tec), DY dyes (Dyomics GmbH), TAMRA (Perkin Elmer), tetramethylrhodamine (TMR), TEXAS RED, DYLIGHT (Thermo Fisher Scientific), FAM (AnaSpec), JOE and ROX (both Applied Biosystems), and Tokyo Green.

[00597] Additional fluorescent dyes which can be used as quenchers includes: DNP, DABSYL, QSY (Molecular Probes), ATTO (Atto-Tec), BHQ (Biosearch Technologies), QXL (AnaSpec), BBQ (Berry and Associates) and CY5Q/7Q (Amersham Biosciences).

[00598] The FRET donor and acceptor moieties which comprise inorganic materials include gold (e.g., quencher), silver, copper, silicon, semiconductor nanoparticles, and fluorescence-emitting metal such as a lanthanide complex, including those of Europium and Terbium.

[00599] Suitable FRET donor/acceptor pairs include: FAM as the donor and JOE, TAMRA, and ROX as the acceptor dyes. Other suitable pairs include: CYA as the donor and R6G, TAMRA, and ROX as the donor dyes. Other suitable donor/acceptor pairs include: a nanoparticle as the donor, and ALEXA FLUORS dyes (e.g., 610, 647, 660, 680, 700). DYOMICS dyes, such as 634 and 734 can be used as energy transfer acceptor dyes.

[00600] The compositions, methods, systems and kits of the present disclosure have particular use in single molecule sequencing reactions. Typically, such applications comprise the performance of a polymerase reaction using the a conjugate comprising a polymerase linked to a label and having polymerase activity according to the present disclosure.

[00601] In one exemplary embodiment, the temporal order of nucleotide incorporations during the polymerase reaction is detected and monitored in real time based on detection of FRET signals resulting from FRET between the labeled polymerase conjugates and the nucleotide label of an incorporating acceptor-labeled nucleotide.

[00602] In some embodiments, the polymerase is linked to a FRET donor and contacted with a nucleotide comprising a FRET acceptor. In some embodiments, the donor performs FRET with the acceptor when the polymerase and nucleotide are brought into sufficient proximity (for example, during a productive incorporation, a non-productive incorporation or during association of a nucleotide with the polymerase active site), resulting in the emission of a FRET signal. The FRET signal can optionally be detected and analyzed to determine the occurrence of a polymerase-nucleotide interaction.

[00603] In some embodiments, the FRET can occur prior to, during or after productive incorporation of the nucleotide into a nucleic acid molecule. Alternatively, the FRET can occur prior to binding of the nucleotide to the polymerase active site, or while the nucleotide resides within the polymerase active site, during a non-productive incorporation.

[00604] In some embodiments, the FRET acceptor moiety can in some embodiments be attached to, or comprise part of, the nucleotide sugar, the nucleobase, or analogs thereof. In some embodiments, the FRET acceptor is attached to a phosphate group of the nucleotide that is cleaved and released upon incorporation of the underlying nucleotide into the primer strand, for example the γ -phosphate, the β -phosphate or some other terminal phosphate of the incoming nucleotide. When this acceptor-labeled nucleotide polyphosphate is incorporated by the labeled polymerase conjugate into a nucleic acid molecule, the polymerase cleaves the bond between the alpha and beta phosphate, thereby releasing a pyrophosphate moiety comprising the acceptor that diffuses away. Thus, in these embodiments, a signal indicative of nucleotide incorporation is generated through FRET between the nanoparticle and the acceptor bonded to the gamma, beta or other terminal phosphate as each incoming nucleotide is incorporated into the newly synthesized strand. By releasing the label upon incorporation, successive incorporation of labeled nucleotides can each be detected without interference from nucleotides previously incorporated into the complementary strand. Alternatively, the nucleotide may be labeled with a FRET acceptor moiety on an internal phosphate, for example, the alpha phosphate, the beta phosphate, or another internal phosphate. Although such alpha-phosphate adducts are not cleaved and released during the polymerization process, they can be removed and/or rendered inoperable through appropriate treatments, e.g., chemical cleavage or photobleaching, later in the sequencing process.

[00605] The polymerase reaction conditions can comprise any suitable reaction conditions that permit nucleotide polymerization by labeled polymerase conjugates of the present disclosure. In one non-limiting example of nucleotide polymerization, the steps of polymerization can comprise: (1) complementary base-pairing of a target DNA molecule (e.g., a template molecule) with a primer molecule having a terminal 3' OH (the terminal 3' OH provides the polymerization initiation site for the

polymerase); (2) binding of the polymerase of the conjugate to the base-paired target DNA/primer duplex to form a complex (e.g., open complex); (3) binding of the candidate nucleotide by the polymerase of the conjugate, which polymerase interrogates the candidate nucleotide for complementarity with the template nucleotide on the target DNA molecule; (4) catalysis of nucleotide polymerization by the polymerase of the conjugate.

[00606] In one embodiment, the polymerase of the conjugate comprises cleavage of the incorporating nucleotide by the polymerase, accompanied by liberation of a nucleotide cleavage product. When the nucleotide is a phosphate-comprising nucleotide, the cleavage product can include one or more phosphate groups. In other embodiments, where the polymerase incorporates a nucleotide analog having substituted phosphate groups, the cleavage product may include one or more substituted phosphate groups.

[00607] The candidate nucleotide may or may not be complementary to the template nucleotide on the target molecule. The candidate nucleotide may dissociate from the polymerase. If the candidate nucleotide dissociates from the polymerase, it can be liberated; in some embodiments, the liberated nucleotide carries intact polyphosphate groups. When the candidate nucleotide dissociates from the DNA polymerase, the event is known as a “non-productive binding” event. The dissociating nucleotide may or may not be complementary to the template nucleotide on the target molecule.

[00608] The incorporated nucleotide may or may not be complementary to the template nucleotide on the target. When the candidate nucleotide binds the DNA polymerase and is incorporated, the event is a “productive binding” event. The incorporated nucleotide may or may not be complementary to the template nucleotide on the target molecule.

[00609] The length of time, frequency, or duration of the binding of the complementary candidate nucleotide to the polymerase can differ from that of the non-complementary candidate nucleotide. This time difference can be used to distinguish between the complementary and non-complementary nucleotides, and/or can be used to identify the incorporated nucleotide, and/or can be used to deduce the sequence of the target molecule.

[00610] The signal (or change in signal) generated by the energy transfer donor and/or acceptor can be detected before, during, and/or after any nucleotide incorporation event.

[00611] In some embodiments, the polymerase reaction includes RNA polymerization which does not require a 3' polymerization initiation site. Polymerase reactions involving RNA polymerization are well known in the art.

[00612] Productive and Non-Productive Binding

[00613] Also provided herein are energy transfer compositions and methods for distinguishing between the productive and non-productive binding events. The compositions and methods can also provide base identity information during nucleotide incorporation. The compositions include nucleotides and polymerases each attached to a energy transfer moiety.

[00614] The compositions and methods provided herein can be used to distinguish events such as productive and non-productive nucleotide binding to the polymerase. In a productive binding event, the nucleotide can bind/associate with the polymerase for a time period which is distinguishable (e.g., longer or shorter time period), compared to a non-productive binding event. In a non-productive binding event, the nucleotide can bind/associate with the polymerase and then dissociate. The donor and acceptor energy transfer moieties produce detectable signals when they are in proximity to each other and can be associated with productive and non-productive binding events. Thus, the time-length difference between signals from the productive and non-productive binding events can provide distinction between the two types of events.

[00615] The detectable signals can be classified into true positive and false positive signals. For example, the true positive signals can arise from productive binding in which the nucleotide binds the polymerase and is incorporated. The incorporated nucleotide can be complementary to the template nucleotide. In another example, the false positive signals can arise from different binding events, including: non-specific binding, non-productive binding, and any event which brings the energy transfer donor and acceptor into sufficient proximity to induce a detectable signal.

[00616] Optionally, polymerase reactions performed using the methods, systems, compositions and kits of the present disclosure can be performed under any

conditions which are suitable for: forming the complex (target /polymerase or target/initiation site/polymerase); binding the nucleotide to the polymerase; permitting the energy transfer and reporter moieties to generate detectable signals when the nucleotide binds the polymerase; incorporating the nucleotide; permitting the energy transfer and reporter moieties to generate a signal upon close proximity and/or nucleotide incorporation; and/or detecting the signal, or change in the signal, from the energy transfer or reporter moieties. The suitable conditions include well known parameters for time, temperature, pH, reagents, buffers, reagents, salts, co-factors, nucleotides, target DNA, primer DNA, enzymes such as nucleic acid-dependent polymerase, amounts and/or ratios of the components in the reactions, and the like. The reagents or buffers can include a source of monovalent ions, such as KCl, K-acetate, NH₄-acetate, K-glutamate, NH₄Cl, or ammonium sulfate. The reagents or buffers can include a source of divalent ions, such as Mg²⁺ and/or Mn²⁺, MgCl₂, or Mg-acetate. The buffer can include Tris, Tricine, HEPES, MOPS, ACES, or MES, which can provide a pH range of about 5.0 to about 9.5. The buffer can include chelating agents such as EDTA and EGTA, and the like.

[00617] Reducing photo-damage

[00618] The suitable polymerase reaction conditions can also include compounds which reduce photo-damage. For example, the compounds may reduce oxygen-damage or photo-damage. Illuminating the nucleotide binding and/or nucleotide incorporation reactions with electromagnetic radiation at an excitation wavelength can induce formation of reactive oxygen species from the fluorophore or other components in the reaction. The reactive oxygen species can cause photo-damage to the fluorophores, polymerases, or any other component of the binding or incorporation reactions. The nucleotide binding or nucleotide incorporation reactions can include compounds which are capable of reducing photo-damage, including: protocatechuate-3,4-dioxygenase, protocatechuic acid; 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (TROLOX); or cyclooctatetraene (COT).

[00619] Other compounds for reducing photo-damage include: ascorbic acid, astaxanthin, bilirubin, biliverdin, bixin, captopril, canthaxanthin, carotene (alpha, beta, and gamma), cysteine, beta-dimethyl cysteine, N-acetyl cysteine, diazobicyclooctane (DABCO), dithiothreitol (DTT), ergothioneine, glucose

oxidase/catalase (GO/Cat), glutathione, glutathione peroxidase, hydrazine (N_2H_4), hydroxylamine, lycopene, lutein, polyene dialdehydes, melatonin, methionine, mercaptopropionylglycine, 2-mercaptoethane sulfonate (MESNA), pyridoxine and its derivatives, mercaptoethylamine (MEA), β -mercaptoethanol (BME), n-propyl gallate, p-phenylenediamine (PPD), hydroquinone, sodium azide (NaN_3), sodium sulfite (Na_2SO_3), superoxide dismutase, tocopherols, α -tocopheryl succinate and its analogs, and zeaxanthin.

[00620] Also provided herein are methods of using the labeled biomolecule conjugates of the present disclosure.

[00621] For example, disclosed herein are methods for incorporation of one or more nucleotides onto the end of a nucleic acid molecule, comprising: contacting a conjugate including a polymerase linked to a label with a nucleotide under conditions where the nucleotide is incorporated into a nucleic acid molecule by the conjugate. The nucleic acid molecule can be any suitable target nucleic acid molecule of interest. In some embodiments, the labeled polymerase can be a polymerase having or comprising the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 or SEQ ID NO: 36, or any sequence at least 80% identical thereto. In some embodiments, the nucleotide can be become incorporated onto the 3' end of an extending nucleic acid molecule by the polymerase. In some embodiments, the nucleotide can be a labeled nucleotide analog. The labeled nucleotide analog can further comprise a label linked to the base, sugar, phosphate or any other portion of the nucleotide analog. In some embodiments, the nucleotide can also comprise a blocking group that inhibits, slows down or blocks further incorporation of nucleotides onto the end of the nucleic acid molecule until the blocking group is removed from the nucleotide. In some embodiments, the nucleotide comprising a blocking group is a reversible terminator for nucleic acid synthesis, as described further below. In some embodiments, the blocking group can be removed from the nucleotide by chemical, enzymatic, or photocleaving reactions.

[00622] In some embodiments, the method further includes the step of adding one or more divalent cations to the polymerase reaction mixture in an amount sufficient for inhibiting further incorporation of nucleotides onto the end of the nucleic acid molecule by the labeled polymerase. In some embodiments, the divalent cation that

inhibits nucleotide incorporation is calcium. In another embodiment, omitting, reducing, or chelating cations that permit nucleotide incorporation (e.g, manganese and/or magnesium) can be employed. Such methods are described, for example, in U.S. Provisional Application 61/242,762, filed September 15, 2009; and in U.S. Provisional Application No. 61/184,774, filed on June 5, 2009. In some embodiments, the polymerase can be linked to a label, as, for example, disclosed herein and in U.S. Provisional Application No. 61/184,770, filed June 5, 2009.

[00623] Also provided herein is a method for detecting one or more nucleotide incorporations, comprising: contacting a conjugate including a polymerase linked to a label with a labeled nucleotide under conditions where the labeled nucleotide is incorporated by the conjugate into a nucleic acid molecule, and where the label of the labeled nucleotide emits a signal indicative of such nucleotide incorporation; and detecting the signal indicative of such nucleotide incorporation. In some embodiments, the detecting can be performed in real or near real time. In some embodiments, the method can further include analyzing the detected signal indicative of nucleotide incorporation to determine the identity of the incorporated nucleotide. In some embodiments, the labeled polymerase conjugate catalyzes a time series of nucleotide incorporations, which can collectively be detected and analyzed to determine some or all of the sequence of the target nucleic acid molecule.

[00624] Also disclosed herein is a method for determining a nucleotide sequence of a single nucleic acid molecule, comprising: (a) conducting a polymerase reaction comprising a labeled biomolecule conjugate and a labeled nucleotide under conditions where the conjugate incorporates the labeled nucleotide into a nucleic acid molecule and a signal indicative of such nucleotide incorporation is generated; (b) detecting the signal indicative of such nucleotide incorporation; and (c) analyzing the signal to determine the identity of the incorporated nucleotide. Optionally, a time series of nucleotide incorporation signals can be detected and analyzed, thereby determining some or all of the nucleotide sequence of a single nucleic acid molecule.

[00625] Also provided herein are methods of sequencing a nucleic acid molecule, comprising: (a) performing a polymerase reaction comprising a labeled polymerase conjugate and labeled nucleotides under conditions resulting in a series of labeled nucleotide incorporations by the polymerase and the generation of a signal indicative

of each nucleotide incorporation the series; (b) detecting a time sequence of nucleotide incorporations; and (c) determining the identity of one or more incorporated nucleotides, thereby determining some or all of the nucleotide sequence of a single nucleic acid molecule.

[00626] In some embodiments, the polymerase is attached to or associated with a substrate or surface. In some embodiments, the polymerase can be attached to or associated with a nucleic acid molecule (termed a template), and polymerize one or more nucleotides in a template-dependent fashion. In some embodiments, the template can be attached to or associated with a substrate or surface. In some embodiments, the polymerase, template, nucleotide, substrate or surface, or some combination thereof, can also be labeled.

[00627] In some embodiments, the methods of the present disclosure can be performed in multiplex and/or “high-throughput” format wherein multiple units of the labeled polymerase conjugates of the present disclosure can each be visualized and monitored in parallel with each other. For example, in some embodiments, multiple labeled polymerase conjugates may be positioned, associated with, or attached to different locations on a substrate, and a polymerase activity of one or more of these polymerases may be detected in isolation. In some embodiments, the polymerase or the template nucleic acid molecule are associated with or attached to a substrate or surface in array format. The array can be spatially addressable.

[00628] In some embodiments, the sequencing reaction can be performed using buffer conditions comprising 50 mM Tris buffer pH 7.5, 50 mM NaCl, 0-10 mM $MgCl_2$, 2 mM $MnCl_2$, 330 nM polymerase, 100nM primed template and 4 μM labeled nucleotide hexaphosphate. Optionally, 0.3% BSA and/or 0.05% Tween20 can be included in the reaction mix. In some embodiments, the reaction mix is further supplemented with 2mM DTT and/or single stranded binding protein (SSBP) at a concentration of 100 $\mu g/ml$.

[00629] Alternatively, in some embodiments the sequencing reaction can be performed using buffer conditions comprising 50mM Tris pH 8.0, 50mM NaCl and 10mM $MgCl_2$.

[00630] In one exemplary embodiment, a nucleic acid sequencing system can comprise a template nucleic acid molecule attached to a substrate, a labeled

polymerase conjugate comprising a FRET donor label linked to a polymerase, and labeled nucleotides each comprising a nucleotide linked to one or more FRET acceptor labels.

[00631] The template nucleic acid molecule of this sequencing system can be attached to any suitable substrate or surface using any suitable method. In some embodiments, the template nucleic acid molecule can comprise one or more biotin moieties, the surface can comprise an avidin moiety, and the template nucleic acid is linked to the surface via one or more biotin-avidin bonds. In some embodiments, the template and surface can each comprise one or more biotin moieties, and be linked to each other through a linkage comprising an avidin moiety.

[00632] An exemplary sequencing system according to the present disclosure is depicted in FIG. 1. This exemplary system comprises a biotinylated nucleic acid template (here, a hairpin oligonucleotide) linked to a surface through one or more biotin-avidin bonds, a labeled polymerase conjugate comprising biotinylated Phi-29 linked to dye-labeled streptavidin, and acceptor-labeled nucleotides. This exemplary system was used to generate the sequence data depicted in FIG. 14 and FIG. 15, according to the methods described in Example 6.

[00633] Polymerization Initiation Sites

[00634] In some embodiments, the polymerase of the labeled polymerase conjugate initiates polymerization at a polymerization initiation site. In some embodiments, the polymerization initiation site can be a terminal 3' OH group of a nucleic acid molecule. The 3' OH group can serve as a substrate for the polymerase for nucleotide polymerization. The 3' OH group can serve as a substrate for the polymerase to form a phosphodiester bond between the terminal 3' OH group and an incorporated nucleotide. The 3' OH group can be provided by: the terminal end of a primer molecule; a nick or gap within a nucleic acid molecule (e.g., oligonucleotide) which is base-paired with the target molecule; the terminal end of a secondary structure (e.g., the end of a hairpin-like structure); or an origin of replication.

[00635] In some embodiments, the polymerization initiation site can be provided by an accessory protein (e.g., RNA polymerase or helicase/primase). The polymerization initiation site can be provided by a terminal protein which can be bound (covalently or non-covalently) to the end of the target nucleic, including

terminal protein (e.g., TP) found in phage (e.g., TP from phi29 phage). Thus, the polymerization initiation site may be at a terminal end or within a base-paired nucleic acid molecule.

[00636] In other embodiments, the polymerization initiation site used by some polymerases (e.g., RNA polymerase) may not include a 3'OH group.

[00637] The portion of the target molecule which is base paired with the primer or with the oligonucleotide, or the self-primed portion of the target molecule, can form hydrogen bonding by Watson-Crick or Hoogsteen binding to form a duplex nucleic acid structure. The primer, oligonucleotide, and self-priming sequence may be complementary, or partially complementary, to the nucleotide sequence of the target molecule. The complementary base pairing can be the standard A-T or C-G base pairing, or can be other forms of base-pairing interactions.

[00638] **Primer Molecules**

[00639] In some embodiments, the primer molecule can hybridize with the target nucleic acid molecule. The sequence of the primer molecule can be complementary or non-complementary with the sequence of the sequence of the target molecule. The 3' terminal end of the primer molecule can provide the polymerization initiation site.

[00640] Optionally, the primers can be modified with a chemical moiety to protect the primer from serving as a polymerization initiation site or as a restriction enzyme recognition site. The chemical moiety can be a natural or synthetic amino acid linked through an amide bond to the primer.

[00641] The primer, oligonucleotide, or self-priming portion, may be naturally-occurring, or may be produced using enzymatic or chemical synthesis methods. The primer, oligonucleotide, or self-priming portion may be any suitable length including 5, 10, 15, 20, 25, 30, 40, 50, 75, or 100 nucleotides or longer in length. The primer, oligonucleotide, or self-priming portion may be linked to an energy transfer moiety (e.g., donor or acceptor) or to a reporter moiety (e.g., a dye) using methods well known in the art.

[00642] The primer molecule, oligonucleotide, and self-priming portion of the target molecule, may comprise ribonucleotides, deoxyribonucleotides, ribonucleotides, deoxyribonucleotides, peptide nucleotides, modified phosphate-sugar backbone nucleotides including phosphorothioate and phosphoramidate, metallonucleosides,

phosphonate nucleosides, and any analogs or variants thereof, or combinations thereof.

[00643] In one embodiment, the primer molecule can be a recombinant DNA molecule. The primer can be linked at the 5' or 3' end, or internally, with a binding partner, such as biotin. The biotin can be used to immobilize the primer molecule to the surface (via an avidin-like molecule), or for attachment to a reporter moiety. The primer can be linked to a energy transfer moiety, such as a fluorescent dye or a nanoparticle, or to a reporter moiety. The primer molecule can hybridize to the target nucleic acid molecule. The primer molecule can be used as a capture probe to immobilize the target molecule.

[00644] The compositions, methods, systems, apparatuses and kits disclosed herein can be practiced using nucleotides. In some embodiments, the nucleotides can be linked with at least one energy transfer moiety. The energy transfer moiety can be an energy transfer acceptor moiety. The different types of nucleotides (e.g., adenosine, thymidine, cytidine, guanosine, and uridine) can be labeled with different energy transfer acceptor moieties so that the detectable signals from each of the different types nucleotides can be distinguishable to permit base identity. The nucleotides can be labeled in a way that does not interfere with the events of polymerization. For example the attached energy transfer acceptor moiety does not interfere with nucleotide binding and/or does not interfere with nucleotide incorporation and/or does not interfere with cleavage of the phosphodiester bonds and/or does not interfere with release of the polyphosphate product. See for example, U.S. Serial No. 61/164,091, Ronald Graham, concurrently filed March 27, 2009. See for example U.S. Patent Nos. 7,041,812, 7,052,839, 7,125,671, and 7,223,541; U.S. Pub. Nos. 2007/072196 and 2008/0091005; Sood et al., 2005, J. Am. Chem. Soc. 127:2394-2395; Arzumanov et al., 1996, J. Biol. Chem. 271:24389-24394; and Kumar et al., 2005, Nucleosides, Nucleotides & Nucleic Acids, 24(5):401-408.

[00645] In one aspect, the energy transfer acceptor moiety may be linked to any position of the nucleotide. For example, the energy transfer acceptor moiety can be linked to any phosphate group (or derivatized phosphate group), the sugar or the base. In another example, the energy transfer moiety can be linked to any phosphate group (or derivatized phosphate group) which is released as part of a phosphate cleavage

product upon incorporation. In yet another example, the energy transfer acceptor moiety can be linked to the terminal phosphate group (or derivatized phosphate group). In another aspect, the nucleotide may be linked with an additional energy transfer acceptor moiety, so that the nucleotide is attached with two or more energy transfer acceptor moieties. The additional energy transfer acceptor moiety can be the same or different as the first energy transfer acceptor moiety. In one embodiment, the energy transfer acceptor moiety can be a FRET acceptor moiety.

[00646] In one aspect, the nucleotide may be linked with a reporter moiety which is not an energy transfer moiety. For example, the reporter moiety can be a fluorophore.

[00647] In one aspect, the energy transfer acceptor moieties and/or the reporter moiety can be attached to the nucleotide via a linear or branched linker moiety. An intervening linker moiety can connect the energy transfer acceptor moieties with each other and/or to the reporter moiety, any combination of linking arrangements.

[00648] In another aspect, the nucleotides comprise a sugar moiety, base moiety, and at least three, four, five, six, seven, eight, nine, ten, or more phosphate groups linked to the sugar moiety by an ester or phosphoramidate linkage. The phosphates can be linked to the 3' or 5' C of the sugar moiety. The nucleotides can be incorporated and/or polymerized into a growing nucleic acid strand by a naturally occurring, modified, or engineered nucleic acid dependent polymerase.

[00649] In one aspect, different linkers can be used to operably link the different nucleotides (e.g., A, G, C, or T/U) to the energy transfer moieties or reporter moieties. For example, adenosine nucleotide can be attached to one type of energy transfer moiety using one type of linker, and guanosine nucleotide can be linked to a different type of energy transfer moiety using a different type of linker. In another example, adenosine nucleotide can be attached to one type of energy transfer moiety using one type of linker, and the other types of nucleotides can be attached to different types of energy transfer moieties using the same type of linker. One skilled in the art will appreciate that many different combinations of nucleotides, energy transfer moieties, and linkers are possible.

[00650] In one aspect, the distance between the nucleotide and the energy transfer moiety can be altered. For example, the linker length and/or number of phosphate groups can lengthen or shorten the distance from the sugar moiety to the energy

transfer moiety. In another example, the distance between the nucleotide and the energy transfer moiety can differ for each type of nucleotide (e.g., A, G, C, or T/U).

[00651] In another aspect, the number of energy transfer moieties which are linked to the different types of nucleotides (e.g., A, G, C, or T/U) can be the same or different. For example: A can have one dye, and G, C, and T have two; A can have one dye, C has two, G has three, and T has four; A can have one dye, C and G have two, and T has four. One skilled in the art will recognize that many different combinations are possible.

[00652] In another aspect, the concentration of the labeled nucleotides used to conduct the nucleotide binding or nucleotide incorporation reactions can be about 0.0001 nM-1 μ M, or about 0.0001 nM-0.001 nM, or about 0.001 nM-0.01 nM, or about 0.01 nM-0.1 nM, or about 0.1 nM-1.0 nM, or about 1 nM-25 nM, or about 25 nM-50 nM, or about 50 nM-75 nM, or about 75 nM-100 nM, or about 100 nM-200 nM, or about 200 nM-500 nM, or about 500 nM-750 nM, or about 750 nM-1000 nM, or about 0.1 μ M-20 μ M, or about 20 μ M-50 μ M, or about 50 μ M-75 μ M, or about 75 μ M-100 μ M, or about 100 μ M-200 μ M, or about 200 μ M-500 μ M, or about 500 μ M-750 μ M, or about 750 μ M-1000 μ M.

[00653] In another aspect, the concentration of the different types of labeled nucleotides, which are used to conduct the nucleotide binding or incorporation reaction, can be the same or different from each other.

[00654] Sugar Moieties

[00655] The nucleotides typically comprise suitable sugar moieties, such as carbocyclic moieties (Ferraro and Gotor 2000 Chem. Rev. 100: 4319-48), acyclic moieties (Martinez, et al., 1999 Nucleic Acids Research 27: 1271-1274; Martinez, et al., 1997 Bioorganic & Medicinal Chemistry Letters vol. 7: 3013-3016), and other suitable sugar moieties (Joeng, et al., 1993 J. Med. Chem. 36: 2627-2638; Kim, et al., 1993 J. Med. Chem. 36: 30-7; Eschenmosser 1999 Science 284:2118-2124.; and U.S. Pat. No. 5,558,991). The sugar moiety may be selected from the following: ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl, 2',3'-dideoxyribosyl, 2',3'-didehydrideoxyribosyl, 2'-alkoxyribosyl, 2'-azidoribosyl, 2'-aminoribosyl, 2'-fluororibosyl, 2'-mercaptoriboxyl, 2'-alkylthioribosyl, 3'-alkoxyribosyl, 3'-azidoribosyl, 3'-aminoribosyl, 3'-fluororibosyl, 3'-mercaptoriboxyl, 3'-alkylthioribosyl carbocyclic,

acyclic and other modified sugars. In one aspect, the 3'-position has a hydroxyl group, for strand/chain elongation.

[00656] Base Moieties

[00657] The nucleotides can include a hetero cyclic base which includes substituted or unsubstituted nitrogen-containing parent heteroaromatic ring which is commonly found in nucleic acids, including naturally-occurring, substituted, modified, or engineered variants. The base is capable of forming Watson-Crick and/or Hoogsteen hydrogen bonds with an appropriate complementary base. Exemplary bases include, but are not limited to, purines and pyrimidines such as: 2-aminopurine, 2,6-diaminopurine, adenine (A), ethenoadenine, N⁶-Δ²-isopentenyladenine (6iA), N⁶-Δ²-isopentenyl-2-methylthioadenine (2ms6iA), N⁶-methyladenine, guanine (G), isoguanine, N²-dimethylguanine (dmG), 7-methylguanine (7mG), 2-thiopyrimidine, 6-thioguanine (6sG), hypoxanthine and O⁶-methylguanine; 7-deaza-purines such as 7-deazaadenine (7-deaza-A) and 7-deazaguanine (7-deaza-G); pyrimidines such as cytosine (C), 5-propynylcytosine, isocytosine, thymine (T), 4-thiiothymine (4sT), 5,6-dihydrothymine, O⁴-methylthymine, uracil (U), 4-thiouracil (4sU) and 5,6-dihydrouracil (dihydrouracil; D); indoles such as nitroindole and 4-methylindole; pyrroles such as nitropyrrole; nebularine; inosines; hydroxymethylcytosines; 5-methylcytosines; base (Y); as well as methylated, glycosylated, and acylated base moieties; and the like. Additional exemplary bases can be found in Fasman, 1989, in: *Practical Handbook of Biochemistry and Molecular Biology*, pp. 385-394, CRC Press, Boca Raton, Florida, and the references cited therein.

[00658] Examples of nucleotides include ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotides, modified peptide nucleotides, metallonucleosides, phosphonate nucleosides, and modified phosphate-sugar backbone nucleotides, and any variants of the foregoing.

[00659] Phosphate Groups

[00660] The nucleotides can optionally include phosphate groups which can be linked to the 2', 3' and/or 5' position of the sugar moiety. The phosphate groups include analogs, such as phosphoramidate, phosphorothioate, phosphorodithioate, and

O-methylphosphoroamidite groups. In one embodiment, at least one of the phosphate groups can be substituted with a fluoro and/or chloro group. The phosphate groups can be linked to the sugar moiety by an ester or phosphoramidate linkage. Typically, the nucleotide comprises three, four, five, six, seven, eight, nine, ten, or more phosphate groups linked to the 5' position of the sugar moiety.

[00661] The disclosed compositions and methods can be practiced using any nucleotide which can be incorporated by a polymerase, including naturally-occurring or recombinant polymerases. In one embodiment, the nucleotides can include a nucleoside linked to a chain of 1 – 10 phosphorus atoms. The nucleoside can include a base (or base analog) linked to a sugar (or sugar analog). The phosphorus chain can be linked to the sugar, for example linked to the 5' position of the sugar. The phosphorus chain can be linked to the sugar with an intervening O or S. In one embodiment, one or more phosphorus atoms in the chain can be part of a phosphate group having P and O. In another embodiment, the phosphorus atoms in the chain can be linked together with intervening O, NH, S, methylene, substituted methylene, ethylene, substituted ethylene, CNH_2 , $\text{C}(\text{O})$, $\text{C}(\text{CH}_2)$, CH_2CH_2 , or $\text{C}(\text{OH})\text{CH}_2\text{R}$ (where R can be a 4-pyridine or 1-imidazole). In one embodiment, the phosphorus atoms in the chain can have side groups having O, BH_3 , or S. In the phosphorus chain, a phosphorus atom with a side group other than O can be a substituted phosphate group. In the phosphorus chain, phosphorus atoms with an intervening atom other than O can be a substituted phosphate group. Some examples of nucleotides are described in Xu, U.S. Patent No. 7,405,281.

[00662] In some embodiments, the nucleotide is a dye-labeled nucleotide that comprises a polyphosphate chain and a dye moiety linked to the terminal phosphate group. In some embodiments, the dye-labeled nucleotide comprises a dye moiety linked to the terminal phosphate through an alkyl linker. Optionally, the linker comprises a 6-carbon chain and has a reactive amine group, and the dye moiety is linked to the terminal phosphate bond through a covalent bond formed with the amine group of the linker. In some embodiments, the polyphosphate chain comprises 4, 5, 6, 7, 8, 9, 10 or more phosphates. One exemplary dye-labeled nucleotide that can be used in the disclosed methods and systems has the general structure shown in FIG. 21. This structure includes a sugar bonded to a hexaphosphate chain at the 5' carbon

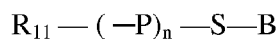
position, and to a nucleotide base (denoted as “N”). The terminal phosphate group of the hexaphosphate is linked to a 6-carbon linker, and the other end of the 6-carbon linker is attached to a dye moiety (denoted as “dye”), typically through an amide bond. In some embodiments, the dye moiety can optionally comprise any one or more of the following dyes: rhodols; resorufins; coumarins; xanthenes; acridines; fluoresceins; rhodamines; erythrins; cyanins; phthalaldehydes; naphthylamines; fluorescamines; benzoxadiazoles; stilbenes; pyrenes; indoles; borapolyazaindacenes; quinazolinones; eosin; erythrosin; Malachite green; CY dyes (GE Biosciences), including Cy3 (and its derivatives) and Cy5 (and its derivatives); DYOMICS and DYLIGHT dyes (Dyomics) including DY-547, DY-630, DY-631, DY-632, DY-633, DY-634, DY-635, DY-647, DY-649, DY-652, DY-678, DY-680, DY-682, DY-701, DY-734, DY-752, DY-777 and DY-782; Lucifer Yellow; CASCADE BLUE ; TEXAS RED; BODIPY (boron-dipyrromethene) (Molecular Probes) dyes including BODIPY 630/650 and BODIPY 650/670; ATTO dyes (Atto-Tec) including ATTO 390, ATTO 425, ATTO 465, ATTO 610 611X, ATTO 610 (N-succinimidyl ester), ATTO 635 (NHS ester); ALEXA FLUORS including ALEXA FLUOR 633, ALEXA FLUOR 647, ALEXA FLUOR 660, ALEXA FLUOR 700, ALEXA FLUOR 750, and ALEXA FLUOR 680 (Molecular Probes); DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one or any derivatives thereof) (Molecular Probes); QUASAR dyes (Biosearch); IRDYES dyes (LiCor) including IRDYE 700DX (NHS ester), IRDYE 800RS (NHS ester) and IRDYE 800CW (NHS ester); EVOBLUE dyes (Evotech Biosystems); JODA 4 dyes (Applied Biosystems); HILYTE dyes (AnaSpec); MR121 and MR200 dyes (Roche); Hoechst dyes 33258 and 33242 (Invitrogen); FAIR OAKS RED (Molecular Devices); SUNNYVALE RED (Molecular Devices); LIGHT CYCLER RED (Roche); EPOCH (Glen Research) dyes including EPOCH REDMOND RED (phosphoramidate), EPOCH YAKIMA YELLOW (phosphoramidate), EPOCH GIG HARBOR GREEN (phosphoramidate); Tokyo green (M. Kamiya, et al., 2005 Angew. Chem. Int. Ed. 44:5439-5441); and CF dyes including CF 647 and CF555 (Biotium).

[00663] In some embodiments, such dye-labeled nucleotides can be used to assay for the nucleotide incorporation kinetics of a particular polymerase according to the procedures described herein (see, e.g., Example 10).

[00664] Non-Hydrolyzable Nucleotides

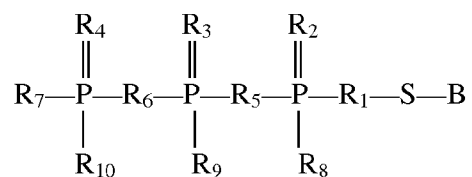
[00665] The nucleotide binding and nucleotide incorporation methods can be practiced using incorporatable nucleotides and non-hydrolyzable nucleotides. In the presence of the incorporatable nucleotides (e.g., labeled), the non-hydrolyzable nucleotides (e.g., non-labeled) can compete for the polymerase binding site to permit distinction between the complementary and non-complementary nucleotides, or for distinguishing between productive and non-productive binding events. In the nucleotide incorporation reaction, the presence of the non-hydrolyzable nucleotides can alter the length of time, frequency, and/or duration of the binding of the labeled incorporatable nucleotides.

[00666] The non-hydrolyzable nucleotides can be non-labeled or can be linked to a reporter moiety (e.g., energy transfer moiety). The labeled non-hydrolyzable nucleotides can be linked to a reporter moiety at any position, such as the sugar, base, or any phosphate (or substituted phosphate group). For example, the non-hydrolyzable nucleotides can have the general structure:



[00667] Where B can be a base moiety, such as a hetero cyclic base which includes substituted or unsubstituted nitrogen-containing heteroaromatic ring. Where S can be a sugar moiety, such as a ribosyl, riboxyl, or glucosyl group. Where n can be 1-10, or more. Where P can be one or more substituted or unsubstituted phosphate or phosphonate groups. Where R₁₁, if included, can be a reporter moiety (e.g., a fluorescent dye). In one embodiment, the non-hydrolyzable nucleotide having multiple phosphate or phosphonate groups, the linkage between the phosphate or phosphonate groups can be non-hydrolyzable by the polymerase. The non-hydrolyzable linkages include, but are not limited to, amino, alkyl, methyl, and thio groups. Non-hydrolyzable nucleotide tetraphosphates having alpha-thio or alpha boreno substitutions having been described (Rank, U.S. published patent application No. 2008/0108082; and Gelfand, U.S. published patent application No. 2008/0293071).

[00668] The phosphate or phosphonate portion of the non-hydrolyzable nucleotide can have the general structure:



[00669] Where B can be a base moiety and S can be a sugar moiety. Where any one of the $R_1 - R_7$ groups can render the nucleotide non-hydrolyzable by a polymerase. Where the sugar C5 position can be CH_2 , CH_2O , $CH=$, CHR , or CH_2CH_2 . Where the R_1 group can be O, S, $CH=$, $CH(CN)$, or NH. Where the R_2 , R_3 , and R_4 groups can independently be O, BH_3 , or SH. Where the R_5 and R_6 groups can independently be an amino, alkyl, methyl, thio group, or CHF, CF_2 , $CHBr$, CCl_2 , O-O, or $-C\equiv C-$. Where the R_7 group can be oxygen, or one or more additional phosphate or phosphonate groups, or can be a reporter moiety. Where R_8 can be SH, BH_3 , CH_3 , NH_2 , or a phenyl group or phenyl ring. Where R_9 can be SH. Where R_{10} can be CH_3 , $N_3CH_2CH_2$, NH_2 , ANS, N_3 , MeO, SH, Ph, F, $PhNH$, PhO , or RS (where Ph can be a phenyl group or phenyl ring, and F can be a fluorine atom or group). The substituted groups can be in the S or R configuration.

[00670] The non-hydrolyzable nucleotides can be alpha-phosphate modified nucleotides, alpha-beta nucleotides, beta-phosphate modified nucleotides, beta-gamma nucleotides, gamma-phosphate modified nucleotides, caged nucleotides, or di-nucleotides.

[00671] Many examples of non-hydrolyzable nucleotides are known (Rienitz 1985 Nucleic Acids Research 13:5685-5695), including commercially-available ones from Jena Bioscience (Jena, Germany).

[00672] In some embodiments, the labeled polymerase conjugates retain polymerase activity. For example, disclosed herein are labeled polymerase conjugates, wherein the polymerase activity of the conjugate can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% relative

to the polymerase activity of the unconjugated enzyme. In a typical assay, the polymerase activity is primer extension activity.

[00673] Various methods of measuring primer extension activity are known in the art. Primer extension activity can be measured using any suitable assay that provides a quantitative indication of the amount of extension product obtained using defined reaction conditions comprising a known concentration of polymerase. Regardless of which assay is used, differences in primer extension activity between two samples, when obtained using identical reaction conditions, can be evaluated by simply comparing levels of observed primer activity obtained from each sample. Optionally, the observed primer extension activity can be normalized for amount of polymerase by dividing the amount of incorporated radioactivity by the polymerase concentration in the reaction mixture, to allow comparison between reactions containing different polymerase concentrations.

[00674] In one exemplary embodiment, the primer extension activity of a polymerase can be measured using a radiometric assay that measures incorporation of a radioactively labeled nucleotide into acid-insoluble material in a polymerase reaction. The amount of incorporated radioactivity indicates the total number of nucleotides incorporated. See, e.g., Wu et al., *Gene Biotechnology*, 2nd Ed., CRC Press; Sambrook, J., Fritsch, EF, and Maniatis, T. (1989) *Molecular Cloning A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[00675] In another exemplary embodiment, levels of primer extension activity in a sample can be measured by monitoring the fluorescence intensity change over time during extension of a fluorescein-labeled hairpin oligonucleotide. Exemplary assays are described in the Examples, herein.

[00676] In yet another exemplary embodiment, the primer extension activity can be quantified by quantifying the amount of pyrophosphate liberated after performing primer extension under defined reaction conditions for 5 minutes.

[00677] In yet another exemplary embodiment, the primer extension activity can be quantified by measuring the fraction of extended primer within a population of primer-template duplexes. In this exemplary embodiment, the template can comprise a radioactive (^{32}P) moiety or fluorescent (TAMRA) label to permit visualization of polymerase reaction products (e.g., extended primer). The primer extension products

can be resolved on a gel, and the primer extension activity can then be quantified as the proportion (%) of extended primer relative to total starting primer, by adding the intensities of all bands observed within a single lane as measured by densitometric analysis.

[00678] When comparing the primer extension activities of conjugates comprising multiple polymerases per conjugate and free (unconjugated) polymerase, the primer extension activities can be normalized for relative polymerase concentration before comparing them against each other. One exemplary such assay is described herein in Example 9.

[00679] In some embodiments, the nucleotide comprises a nucleotide analog that is capable of acting as a reversible terminator of nucleic acid synthesis. Typically, reversible terminators can be incorporated by a polymerase onto the end of an extending nucleic acid molecule, but then “terminate” further synthesis by blocking further addition of nucleotides. In some embodiments, this “termination” capability can be manipulated by adjusting the reaction conditions and/or by suitable treatment. The ability to terminate can result from the presence of a moiety or group, typically named a “blocking” group, which is linked to the nucleotide. In some embodiments, the ability of the nucleotide to terminate nucleic acid synthesis can be eliminated through physical removal, cleavage, structural modification or disruption of the blocking group. The blocking group can be attached to any portion of the nucleotide including, for example, a base moiety, sugar moiety or phosphate moiety. The blocking group can be attached to the nucleotide via a linker. The linkage between the blocking group and the nucleotide can be a photocleavable, chemically cleavable, enzymatically cleavable, thermocleavable (i.e., cleavable upon adjustment of temperature) or pH-sensitive linkage. In some embodiments, the label (which is linked to the nucleotide) is the blocking group.

[00680] In some embodiments, the reversible terminator further comprises a label or tag that facilitates detection of nucleotide. The label can be a fluorescent label. In some embodiments, the label can also be removed via suitable treatment. In some embodiments, the label is released from the nucleotide during incorporation of the nucleotide into the extending nucleic acid molecule. Alternatively, the label becomes incorporated into the extending nucleic acid molecule and is then removed via

suitable treatment. In some embodiments, the label is attached to the nucleotide via a cleavable linkage. The cleavable linkage can be a photocleavable, chemically cleavable, enzymatically cleavable, thermocleavable (i.e., cleavable upon adjustment of temperature) or pH-sensitive linkage.

[00681] The removal of the blocking group can be accomplished in a variety of ways. In some embodiments, the blocking group is attached to the nucleotide via a photocleavable linkage and can be removed from the nucleotide via exposure to photocleaving radiation. In some embodiments, the linkage is a chemically or enzymatically cleavable linkage. In some embodiments, the linkage can be disrupted by varying reaction conditions, e.g., pH, temperature, concentrations of divalent cations, etc.

[00682] Non-limiting examples of suitable reversible terminators include, *inter alia*, nucleotide base-labeled nucleotides comprising one or more blocking groups attached to 3' hydroxyl group, the base moiety or a phosphate group. For example, the nucleotide can comprise an azidomethyl group linked to the 3' hydroxyl group and a fluorescent label linked to the base of the nucleotide. In some embodiments, the reversible terminator can comprise one or more blocking groups attached to the phosphate group. In some embodiments, the nucleotide can comprise a blocking group and a label. In some embodiments, both the blocking group and the label can be linked to the base moiety, while the 3' hydroxyl group is not modified. In some embodiments, the blocking group can be a photocleavable group linked to the base of the nucleotide. See, e.g., U.S. Publication No. 2008/0132692, published June 5, 2008. Further examples of nucleotides comprising extension blocking groups and methods of their use in polymerase-based applications can be found, for example, in U.S. Patent No. 7,078,499 issued July 18, 2006; as well as in U.S. Published Application Nos. 2004/0048300 published March 11, 2004; 2008/0132692 published June 5, 2008; 2009/0081686, published March 26, 2009; and 2008/0131952, published June 5, 2008; Tsien, WO/1991/006678; Stemple, U.S. Patent No. 7,270,951, Balasubramanian, U.S. Patent No. 7,427,673; Milton, U.S. Patent No. 7,541,444.

[00683] In some embodiments, the nucleotide comprises a cleavable label linked to the base. In some embodiments, the blocking group and the label can be removed via

the same cleavage treatment. See, e.g., U.S. Patent No. 7,553,949, issued June 30, 2009. Alternatively, different treatments can be required to remove the blocking group and the label. In some embodiments, the label of the reversible terminator correlates with the base identity of the nucleotide. In some embodiments, each reversible terminator is added sequentially to the polymerase reaction; alternatively, different kinds of reversible terminators can be present simultaneously in the reaction mixture.

[00684] In some embodiments, the blocking group is linked to the 2' hydroxyl group of the sugar moiety. See, e.g., U.S. Patent No. 7,553,949, issued June 30, 2009.

[00685] In some embodiments, the reversible terminator can comprise more than one blocking group. In some embodiments, these multiple blocking groups may function cooperatively by enhancing the termination efficiency of the nucleotide. In one exemplary embodiment, the nucleotide comprises a blocking group linked to the base moiety, while another group linked to the terminal phosphate group further suppresses the incorporation of a nucleotide onto the free 3' hydroxyl group. See, e.g., U.S. Patent Application No. 12/355,487, filed January 16, 2009.

[00686] Typically, the labeled polymerase conjugates of the present disclosure can be used to sequence one or more nucleic acid molecules of interest. In an exemplary method, the reversible terminator is incorporated in a template-dependent manner onto the 3' end of an extending nucleic acid molecule by a labeled polymerase conjugate. The incorporated reversible terminator is detected and identified; and the blocking group of the reversible terminator is then removed. In some embodiments, the unincorporated reversible terminators can be washed away; in some embodiments, it is not necessary to wash or otherwise remove the unincorporated reversible terminators prior to detection, identification or subsequent extension of the extending nucleic acid molecule. In some embodiments, incorporation of the reversible terminator onto the end of a nucleic acid molecule can involve the formation of a covalent bond between the reversible terminator and the nucleotide moiety at the 3' end of the nucleic acid molecule. Alternatively, incorporation of reversible terminator onto the end of a nucleic acid molecule will not involve formation of any covalent bond between the reversible terminator and the nucleotide moiety at the 3' end of the nucleic acid molecule; instead, the reversible terminator is

bound in a template-dependent fashion and positioned within the active site of the polymerase until the blocking group is cleaved or otherwise removed, following which the remaining portion of the reversible terminator can remain as a portion of the extending nucleic acid molecule or alternatively will also dissociate from the polymerase active site and diffuse away.

[00687] In some embodiments, the nucleic acid molecule, the polymerase, or both, may be isolated within a suitable nanostructure. In some embodiments, the nanostructure can be useful in elongating the nucleic acid molecule to permit visualization of nucleotide synthesis along some or all of the length of the nucleic acid molecule. In some embodiments, the nanostructure is also useful in limiting the amount of background signal ("noise") in the system by reducing the excitation or detection volume, and/or by reducing the amount of labeled moieties present within the reaction chamber. In some embodiments, the nanostructure is designed to admit only a single polymeric molecule and elongate it as it flows through the nanostructure. Suitable devices comprising nanostructures that may be used to practice the inventions disclosed herein are described, for example, in U.S. Patent No. 6,635,163; U.S. Patent No. 7,217,562, U.S. Pub. No. 2004/0197843 and U.S. Pub. No. 2007/0020772. In some embodiments, the nanostructures of the nanofluidic device will satisfy three requirements: (1) they will have a sufficiently small dimension to elongate and isolate macromolecules; (2) they will be sufficient length to permit instantaneous observation of the entire elongated macromolecule; and (3) the nanochannels or other nanostructures will be sufficiently numerous to permit simultaneous and parallel observation of a large population of macromolecules. In one embodiment, the radius of the component nanostructures of the nanofluidic device will be roughly equal to or less than the persistence length of the target DNA. Suitable methods of detecting nucleotide incorporations using nanostructures are disclosed, for example, in U.S. Provisional Application Nos. 61/077,090, filed June 30, 2008; 61/089,497, filed August 15, 2008; and 61/090,346, filed August 20, 2008; and International Application No. PCT/US09/49324, filed June 30, 2009.

[00688] **Signal Detection**

[00689] In some embodiments, the label of the disclosed labeled conjugates can emit, or cause to be emitted, a signal that permits visualization of the conjugate and/or provides an indication of biomolecular activity.

[00690] Particular disclosed herein are compositions, methods and systems relating to labeled polymerase conjugates, wherein the conjugate emits, or causes the emission of, a signal indicating a nucleotide incorporation by the polymerase of the conjugate.

[00691] In some embodiments, the signal is an optically detectable signal. Optionally, the optically detectable signal can be a fluorescent signal.

[00692] The signal emitted, or caused to be emitted by the labeled conjugates of the disclosed compositions, methods and systems can be detected and analyzed using any suitable methods and related devices. A wide variety of detectors are available in the art. Representative detectors include but are not limited to optical readers, high-efficiency photon detection systems, photodiodes (e.g. avalanche photo diodes (APD); APD arrays, etc.), cameras, charge couple devices (CCD), electron-multiplying charge-coupled device (EMCCD), intensified charge coupled device (ICCD), photomultiplier tubes (PMT), a multi-anode PMT, and a confocal microscope equipped with any of the foregoing detectors. Where desired, the subject arrays can contain various alignment aides or keys to facilitate a proper spatial placement of each spatially addressable array location and the excitation sources, the photon detectors, or the optical transmission element as described below.

[00693] The systems and methods can detect and/or measure a change or an amount of change of an optical or spectral characteristic of a signal (e.g., fluorescence or quenching) from a label. In some embodiments, the label can be the label of the conjugate or a nucleotide label. The change in the signal can include changes in the: intensity of the signal; duration of the signal; wavelength of the signal; amplitude of the signal; duration between the signals; and/or rate of the change in intensity, duration, wavelength or amplitude. The change in the signal can include a change in the ratio of the change of the energy transfer donor relative to change of the energy transfer acceptor signals.

[00694] In some embodiments, the detection system comprises: excitation illumination, optical transmission elements, detectors, and/or computers.

[00695] The detection system can comprise excitation illumination which can excite the energy transfer or reporter moieties which produce a detectable signal. The excitation illumination can be electromagnetic energy, such as radio waves, infrared, visible light, ultraviolet light, X-rays or gamma rays. The source of the electromagnetic radiation can be a laser, which possesses properties of monochromaticity, directionality, coherence, polarization, and/or intensity. The laser can produce a continuous output beam (e.g., continuous wave laser) or produce pulses of light (e.g., Q-switching or mode-locking). The laser can be used in a one-photon or multi-photon excitation mode. The laser can produce a focused laser beam. The wavelength of the excitation electromagnetic radiation can be between about 325-850 nm, or between about 325-752 nm, or between about 330-752 nm, or between about 405-752 nm. The laser can be generated by a mercury, xenon, halogen, or other lamps.

[00696] The wavelength and/or power of the excitation illumination can be selected to avoid interfering with or damaging the polymerase enzymatic activities. The excitation illumination can be focused on a stationary position or moved to a different field of view (FOV). The excitation illumination can be directed at a nucleotide incorporation reaction which is: in a liquid volume (e.g., aqueous or oil); on a surface; in or on a nanodevice; in a waveguide; or in an evanescent illumination system (e.g., total internal reflection illumination). The excitation illumination can pass through a transparent or partially transparent surface which is conjugated (covalently or non-covalently) with the components of the nucleotide incorporation reaction.

[00697] The energy transfer moiety (e.g., a FRET donor) can be excited by the excitation illumination at a particular wavelength, and transmit the excitation energy to an acceptor moiety which is excited and emits a signal at a longer wavelength. The energy transfer moiety or reporter moiety can undergo multi-photon excitation with a longer wavelength, typically using a pulsed laser.

[00698] The detection system comprises suitable optical transmission elements which are capable of transmitting light from one location to another with the desired refractive indices and geometries. The optical transmission elements transmit the excitation illumination and/or the emitted energy in an unaltered or altered form. The optical transmission elements include: lens, optical fibers, polarization filters (e.g.,

dichroic filters), diffraction gratings (e.g., etched diffraction grating), arrayed waveguide gratings (AWG), optical switches, mirrors, dichroic mirrors, dichroic beam splitter, lenses (e.g., microlens and nanolens), collimators, filters, prisms, optical attenuators, wavelength filters (low-pass, band-pass, or high-pass), wave-plates, and delay lines, or any combination thereof.

[00699] The detection system comprises suitable detectors which are capable of detecting and/or distinguishing the excitation illumination and/or the emitted energy. A wide variety of detectors are available in the art, including: single or multiple channel detectors, high-efficiency photon detection systems, optical readers, charge couple devices (CCD), photodiodes (e.g. avalanche photo diodes (APD)), APD arrays, cameras, electron-multiplying charge-coupled device (EMCCD), intensified charge coupled device (ICCD), photomultiplier tubes (PMT), multi-anode PMT, complementary metal oxide semiconductor (CMOS) chip(s), and a confocal microscope equipped with any of the foregoing detectors. The location of the nucleotide incorporation reaction can be aligned, with respect to the excitation illumination and/or detectors, to facilitate proper optical transmission.

[00700] Suitable detection methods can be used for detecting and/or distinguishing the excitation illumination (or change in excitation illumination) and/or the emitted energy (or change in emitted energy), including: confocal laser scanning microscopy, Total Internal Reflection (TIR), Total Internal Reflection Fluorescence (TIRF), near-field scanning microscopy, far-field confocal microscopy, wide-field epi-illumination, light scattering, dark field microscopy, photoconversion, wide field fluorescence, single and/or multi-photon excitation, spectral wavelength discrimination, evanescent wave illumination, scanning two-photon, scanning wide field two-photon, Nipkow spinning disc, multi-foci multi-photon, or any combinations thereof.

[00701] The signals emitted from different energy transfer moieties can be resolved using suitable discrimination methods which are based on: fluorescence resonance energy transfer measurements; photoconversion; fluorescent lifetime measurements; polarization; fluorescent lifetime determination; correlation/anti-correlation analysis; Raman; intensity; ratiometric; time-resolved methods; anisotropy; near-field or far field microscopy; fluorescence recovery after photobleaching (FRAP); spectral wavelength discrimination; measurement and separation of fluorescence lifetimes;

fluorophore identification; background suppression, parallel multi-color imaging, or any combination thereof. See, for example, J. R. Lakowitz 2006, in: "Principles of Fluorescence Spectroscopy", Third Edition. If the different nucleotides are labeled with different energy transfer or reporter moieties, then resolving the emitted signals can be used to distinguish between the different nucleotides which bind the polymerase and/or which are incorporated by the polymerase.

[00702] In one embodiment, a system and method for detecting radiation emitted by an excited energy transfer or reporter moiety comprises: an illumination source (e.g., a laser) which produces the excitation energy (e.g., one or multi-photon excitation radiation) which is directed, via a dichroic beam splitter, through a lens, and through a transparent surface or onto a surface, where the nucleotide binding reaction or the nucleotide incorporation reaction is attached to the surface or is in a solution. The excitation illumination excites the energy transfer or reporter moiety (e.g., fluorescent dye and/or nanoparticle) resulting in emitted radiation (or a change in radiation) which passes back through the dichroic beam splitter and is directed to the detector (or an array of detectors) which is capable of identifying and/or resolving the type of emission. Information about the detected emitted signals is directed to the computer where the information is registered and/or stored. The computer can process the registered and/or stored information to determine the identity of the nucleotide which bound the polymerase or the identity of the incorporated nucleotide.

[00703] In one aspect, the system and method for detecting radiation emitted by an excited energy transfer or reporter moiety includes a multifuorescence imaging system. For example, the different nucleotides may each be linked to different FRET acceptor moieties. The FRET acceptor moieties can be selected to have minimal overlap between the absorption and emission spectra, and the absorption and emission maxima. The multifuorescence imaging system can simultaneously (or substantially simultaneously) detect signals from the FRET acceptor moieties, and resolve the signals. Such multifuorescent imaging can be accomplished using suitable filters, including: band pass filters, image splitting prisms, band cutoff filters, wavelength dispersion prisms, dichroic mirrors, or diffraction gratings, or any combination thereof.

[00704] In another aspect, the multifluorescence imaging system is capable of detecting the signals emitted by the different energy transfer and reporter moieties attached to the different nucleotides. Such a system can include special filter combinations for each excitation line and/or each emission band. In one embodiment, the detection system includes tunable excitation and/or tunable emission fluorescence imaging. For tunable excitation, light from a light source can pass through a tuning section and condenser prior to irradiating the sample. For tunable emissions, emissions from the sample can be imaged onto a detector after passing through imaging optics and a tuning section. The tuning sections can be controlled to improve performance of the system.

[00705] In yet another aspect, the detection system comprises an optical train which directs signals emitted from an organized array onto different locations of an array-based detector to detect multiple optical signals from multiple locations. The optical trains typically include optical gratings and/or wedge prisms to simultaneously direct and separate signals having differing spectral characteristics from different addressable locations in an array to different locations on an array-based detector, e.g., a CCD.

[00706] In another aspect, the detection methods include detecting photon bursts from the labeled nucleotides during incorporation. The photon bursts can be the fluorescent signals emitted by the energy transfer moiety which is linked to the nucleotide. The photon bursts can be a FRET event. The methods can additionally include analyzing the time trace of the photon bursts. The methods can be practiced using time-resolved fluorescence correlation spectroscopy.

[00707] Nucleotide incorporation reactions using nucleotides labeled at the terminal phosphate with a fluorescent dye have been previously demonstrated (Sood, U.S. published patent application No. 2004/0152119; and Kumar, U.S. Patent No. 7,393,640). Furthermore, fluorescence detection of single molecule nucleotide incorporation reactions has been routinely obtained (Kao, U.S. Patent No. 6,399,335; and Fuller, U.S. Patent No. 7,264,934).

[00708] The nucleotide labeling strategy can be used as a basis for selecting any suitable detection system for detecting and/or resolving signals emitted by the nucleotide binding reaction or the nucleotide incorporation reaction. Exemplary

labeling and detection strategies include but are not limited to optical train and TIRF detection methods such as those disclosed by Harris in U.S. Patent Nos. 6,423,551; and U.S. Pub. Nos. 2006/0176479, 2007/0109536, 2007/0111350, and 2007/0250274.

[00709] Once FRET events have been identified, they can be analyzed to determine the order and sequence of nucleotide incorporations, thereby determining some or all of the sequence of the nucleic acid template that is acted upon by the polymerase. In some embodiments, the FRET events are then computationally filtered to determine the nature of the underlying event and/or to identify the substrate. In another exemplary embodiment, detection events can be analyzed to determine sequence information using the procedure described in Example 6.

[00710] Also provided herein are kits for conducting the nucleotide binding reactions and/or the nucleotide incorporation reactions described herein. The kits can include, in one or more containers, the components of nucleotide binding and/or nucleotide incorporation disclosed herein, including: labeled biomolecule conjugates, labeled polymerase conjugates, nucleotides, target nucleic acid molecules (e.g., a control test target molecules), primers, and/or oligonucleotides.

[00711] In some embodiments, the kit comprises a labeled polymerase conjugate according to the present disclosure. Optionally, the kit can further include a nucleotide. The nucleotide can be a labeled nucleotide. In some embodiments, the nucleotide includes a polyphosphate chain. The nucleotide label can optionally be attached to the terminal phosphate group of the nucleotide.

[00712] In the kits, the solid surfaces, energy transfer moieties, reporter moieties, nanoparticles, polymerases, nucleotides, target nucleic acid molecules, primers, and/or oligonucleotides can be attached to each other in any combination, and/or be unattached. The kits can include positive and/or negative control samples.

[00713] Additional components can be included in the kit, such as buffers and reagents. For example, the buffers can include Tris, Tricine, HEPES, or MOPS, or chelating agents such as EDTA or EGTA. In another example, the reagents can include monovalent ions, such as KCl, K-acetate, NH₄-acetate, K-glutamate, NH₄Cl, or ammonium sulfate. In yet another example, the reagents can include divalent ions, such as Ca²⁺, CaCl₂, Mg²⁺, MgCl₂, Mg-acetate, Mn²⁺, MnCl₂, and the like. The kits

can include the components in pre-measured unit amounts. The kits can include instructions for performing the nucleotide binding reactions and/or the nucleotide incorporation reactions. Where the kit is intended for diagnostic applications, the kits may further include a label indicating regulatory approval for the diagnostic application.

[00714] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of embodiments, these embodiments are in no way intended to limit the scope of the claims, and it will be apparent to those of skill in the art that variations can be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related can be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLES.

[00715] **Example 1: Preparation of dye-labeled polymerase using biotin-avidin binding pairs**

[00716] This Example illustrates the preparation of an exemplary labeled polymerase conjugate comprising streptavidin labeled with a Cy3B dye moiety linked to biotinylated Phi29 polymerase.

[00717] To prepare Cy3B labeled streptavidin, 25 mg of streptavidin were dissolved in 5 ml of water and the solution was filtered through a 0.2 micrometer filter. To this solution was added 2 ml of a freshly prepared 1M sodium bicarbonate, followed by 700 µl of a solution of 5 mg Cy3B NHS ester in DMSO. The solution was mixed and allowed to react at room temperature for 3 hours with occasional mixing. A small amount of precipitate formed and was removed by centrifugation and the resulting mixture was processed through multiple rounds of ultrafiltration using 10,000

MWCO ultrafiltration devices and PBS as the buffer. The ultrafiltration was continued until the filtrate did not show any traces of free dye by absorbance measurements. The resulting solution was characterized by measuring its UV absorbance and the degree of dye loading was determined from the known molar extinction coefficients of the Cy3B and streptavidin. The degree of loading thus calculated was 3.9 dyes/molecule of streptavidin. The product was stored at 4°C.

[00718] The Cy3B labeled streptavidin was conjugated to biotinylated Phi29 polymerase using biotin-avidin binding to generate a linkage comprising one or more biotin-avidin bonds. Briefly, biotinylated Phi29 was obtained by bacterial expression using the AviTag™ system. The resulting biotinylated Phi29 protein is modified by a single biotin residue located at a predetermined site close to the N-terminus of the protein. In addition, the expressed polymerase contains a hexahistidine tag. Conjugation was performed by mixing 150 µl of 85 µM of the biotinylated Phi29 with 250 µl of PBS buffer. To this was added 40 µl of 5M NaCl followed by 250 µl of 94 µM Cy3B labeled streptavidin. The mixture was mixed and allowed to react for 1 hour at 4°C. A small amount of precipitation was removed by centrifugation and then the mixture was loaded onto a 1 ml His-Trap cartridge, pre-equilibrated with PBS buffer. The cartridge was washed with PBS buffer until no more free, dye-labeled streptavidin was eluting, whereupon the desired reaction product was eluted from the column using a solution of 500 mM imidazole in PBS. The product thus obtained was dialyzed overnight against a buffer containing 50 mM TrisHCl pH 7.5, 150 mM NaCl, 5 mM DTT, 0.1% Tween 20, 0.2 mM EDTA and 50% glycerol.

[00719] Example 2: Evaluation of Nucleotide Binding and Discrimination Activity of Dye-Labeled Polymerase

[00720] The ability of a dye-labeled polymerase prepared according to the method of Example 1, above, to discriminate between a “correct” and an “incorrect” incoming, dye-labeled nucleotide polyphosphate when bound to a primed template was evaluated. Solely in the context of this Example, the term “correct” is used to refer to a nucleotide that can undergo Watson-Crick base pairing with the nucleotide of the template that is immediately adjacent to the 3’ end of the primer, whereas “incorrect” refers to any nucleotide that is not capable of undergoing such Watson-Crick base pairing with the nucleotide of the template. In this assay, the template and primers

were selected such that a nucleotide polyphosphate comprising an adenosine as the nucleobase would be the “correct” nucleotide.

[00721] In a typical experiment, serial dilutions of AF647 labeled deoxyguanosine hexaphosphate, wherein the AF647 label is attached to the terminal phosphate group (referred to herein as “omega-labeled AF647-dG6P” or simply as “ ω AF647-dG6P”) were prepared in the wells of a microtiter plate, in a buffer containing 50 mM TrisHCl pH 7.5, 50 mM NaCl and either 2 mM MnCl_2 or 10 mM CaCl_2 . The highest final concentration of the labeled nucleotide was 4 μM . Aliquots of solutions of preformed complexes between a dye-labeled streptavidin-biotin Phi29 and different primer-template duplexes were added. (Note: The primer-template duplexes can be replaced by suitable hairpin-type oligonucleotides comprising a 5' single stranded overhang). In some experiments, the 3' ends of the primer strands included dideoxy-modified residues in order to prevent the enzymatic elongation of the primers by the polymerase.

[00722] The resulting ternary complexes between the dye-labeled polymerase, the primer-template duplex (or alternatively the self-priming hairpin oligonucleotide) and the labeled nucleotide can be detected by measuring the fluorescence resonance energy transfer (FRET) signal between the dye of the dye-labeled polymerase (in this case, Cy3B) and the dye linked to the terminal phosphate group of the nucleotide (in this case AF647). The results from such a binding experiment are shown in FIG. 4. The individual curves depicted in FIG. 4 are: Curve A: “incorrect” nucleotide, Mn^{2+} buffer; Curve B: “incorrect” nucleotide, Ca^{2+} buffer; Curve C: “correct” nucleotide, Mn^{2+} buffer; Curve D: “correct” nucleotide, Ca^{2+} buffer. These results indicate that the affinity of the polymerase for the correct incoming nucleotide is greater than the affinity for the incorrect nucleotide, and that the binding event can be detected by the resulting FRET between the donor and acceptor dyes. In addition, these results indicate that better discrimination can be achieved in a buffer containing Ca^{2+} compared to a buffer containing Mn^{2+} .

[00723] Example 3: Preparation of a dye-labeled polymerase comprising different types of labels

[00724] A labeled polymerase conjugate comprising two different types of dye labels, Alexa Fluor 488 and Cy3B, linked to Phi-29 polymerase was prepared. To

prepare the conjugate, 50 μ l of an 85 μ M solution of biotin Phi29 was added to 180 μ l of 34 μ M AF488 labeled streptavidin solution, followed by 150 ml of PBS buffer. The mixture was left at 40°C for one hour and then loaded onto a His-Trap column. The excess free labeled streptavidin was removed by washing the column with PBS, whereupon a solution of 50 mM Cy3B-biotin was introduced into the column. The excess Cy3B-biotin was washed off with PBS buffer and the conjugate was eluted with 500 mM imidazole in PBS buffer. The resulting product was dialyzed against the same buffer described above. The presence of two different dyes in the final product was confirmed by UV absorbance measurements (data not shown).

[00725] The polymerase activity of the resulting conjugate was evaluated in a nucleic acid binding assay using an AF647-labeled DNA molecule. Serial dilutions of the latter (starting at 1 μ M) were mixed with 120 nM of the resulting AF488/Cy3B-streptavidin-biotin-Phi-29 conjugate. The resulting FRET was measured using two different excitation wavelengths: 490 and 540 nm, with the emission being measured at both 580 and 670 nm. The results are depicted in FIG. 5. The five binding curves depicted in FIG. 5 representing the following: Curve 1: Excitation at 490 nm and emission at 525nm; Curve 2: Excitation at 490 nm and emission at 580 nm; Curve 3: Excitation at 490 nm and emission at 670 nm; Curve 4: Excitation at 540 nm, emission at 580 nm; and Curve 5: Excitation at 540 nm and emission at 670 nm. As depicted in FIG. 5, the use of an excitation wavelength of 490 nm results in substantially higher acceptor emission signals at 670 nm than use of an excitation wavelength at 540 nm, with reduced donor emission (“bleed-through”) in the acceptor channel.

[00726] **Example 4: Preparation of a labeled polymerase conjugate comprising a single label covalently attached to a polymerase**

[00727] 80 μ L of an 111 μ M stock solution of His-tagged polymerase comprising the amino acid sequence of SEQ ID NO: 34 (referred to herein as “HP1-B104 exo-polymerase”) in 10 mM Tris (pH 7.5), 100 mM NaCl, 4 mM DTT, 0.5% v/v Tween-20, 0.1 mM EDTA and 50% v/v glycerol) was buffer exchanged into 1x phosphate buffered saline (1xPBS) pH 7.4 with additional 200 mM NaCl using an NAP-5 column.

[00728] The buffer-exchanged His-tagged polymerase (150 μ L, 18.9 μ M in 1xPBS pH 7.4 with additional 200 mM NaCl) was mixed with 800 μ L of a buffer comprising 1x PBS buffer, pH 7.4 and 200 mM NaCl. The mixture was added into a vial containing 6.5 μ L of 1.3 mM Cy3B NHS ester in DMSO in a 3:1 dye to polymerase molar ratio. The reaction solution was mixed and rotated at 4 °C for 2 hour, and then centrifuged for 5 minutes at 16.8K rcf. The supernatant was loaded by a syringe onto a Ni-NTA cartridge that was previously washed by ~6 mL water and then by ~6 mL of 100 mM Tris buffer (pH 7.5) with 300 mM NaCl and 1 mM DTT. The column was continuously washed with 100 mM Tris buffer (pH 7.5) with 300 mM NaCl and 1 mM DTT to completely remove the unbound dye. The conjugate retained on the column during the wash was eluted from the cartridge by using 100 mM Tris buffer (pH 7.5) with 0.5 M imidazole, 300 mM NaCl and 1 mM DTT. The solution collected from the cartridge was centrifuged and transferred into a 10K MWCO dialysis cassette. The solution was then dialyzed at 4 °C overnight into a buffer comprising 50 mM Tris buffer pH7.5, 150 mM NaCl, 0.2 mM EDTA, 0.1% v/v Tween-20, 5 mM DTT and 50% v/v glycerol. The dye:enzyme stoichiometry of the resulting conjugate preparation was measured using UV absorbance to estimate the concentration of the protein (280 nm) and Cy3B dye (564 nm) and was determined to be about 1 Cy3B dye label per polymerase. This conjugate preparation was assayed to determine concentration, DNA extension activity, and DNA binding by FRET as described below.

[00729] Primer extension assays were performed to measure the primer extension activity of the labeled polymerase conjugates. Primer extension activity is quantified by monitoring the fluorescence intensity change over time during extension of a fluorescein-labeled hairpin oligonucleotide, comprising the following nucleotide sequence, known as "oligo 221" (SEQ ID NO: 43 below). The fluorescence intensity correlates with the level of primer extension activity in the sample.

[00730] The extension buffer used was 50 mM Tris buffer pH 7.5 with 50 mM NaCl, 10 mM MgCl₂ and 0.5 mM MnCl₂.

[00731] To reaction wells containing 100 μ L of 150 nM of a fluorescein-labeled hairpin oligonucleotide, oligo221 (SEQ ID NO: 43), and 10 nM of the labeled

polymerase conjugate in extension buffer, 2 μ L of 1 mM dATP was added to initiate the extension reaction. Oligo 221 comprises the following sequence:

[00732] (5'- TTTTTTGCAGGTGACAGGTTTTCTGTCACC(fluorescein-T)GC-3')

[00733] (SEQ ID NO: 43)

[00734] The fluorescence intensity in the wells was recorded at 525 nm fluorescence with 490 nm excitation for every 20 second in a 10 minutes period immediately after the addition of dATP. Assay control reaction wells contained the same components but no dATP was added.

[00735] To calculate the enzyme activity rate, reference polymerase reaction/control wells were included containing 150 nM fluorescein-labeled oligo-221 and 50 nM free polymerase with 20 μ M dATP (for reaction wells) or without 20 μ M dATP (for control wells) in the same extension buffer as above. The time course data for conjugate reaction/control and reference polymerase reaction/control is used to calculate the conjugate activity rate using the following equations:

$$turnover_rate(base/sec) = \frac{\Delta RFU_{sample_per_sec}}{\Delta RFU_{max_per_nMsubs}} \times \frac{1}{10nM} \times 7(base)$$

$$\text{and } \Delta RFU_{max_per_nMsubs} = \frac{RFU_{max} - RFU_{min}}{substr_conc.(nM)}$$

[00736] Where: RFU_{max} is the average maximal RFU in the reference polymerase reaction wells;

[00737] RFU_{min} is the average minimal RFU in the reference polymerase control wells;

[00738] Substr_conc. (nM) is the oligo 221 concentration in assay, which is 150 nM.

$$\Delta RFU_{sample_per_sec} = \frac{RFU_t - RFU_0}{t(sec)}$$

[00739] Where: t (sec) is the time period where the fluorescence intensity increases in the conjugate reaction well linearly from the start;

[00740] RFU_t is the average RFU of the conjugate extension wells for the tested sample at t second point; and

[00741] RFU_0 is the average RFU of the conjugate extension wells for the tested sample at the start point.

[00742] Representative results of extension assays comparing the extension observed using the conjugate of this Example and the conjugate of the next Example, as well as using a control (unconjugated) polymerase, are depicted in FIG. 6.

[00743] In addition, FRET-based binding assays were performed to evaluate the ability of the conjugates to bind to a nucleic acid template. The assay buffer is 50 mM Tris buffer pH 7.5 with 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM $MnCl_2$. 50 μ L of 20 nM conjugate was added into each well that contains either 50 μ L of ALEXA FLUOR 647 labeled oligonucleotide 199 or 50 μ L of ALEXA FLUOR 647 labeled oligonucleotide 192 at various concentrations (2-fold dilution series with concentrations ranging from 1000 nM to 0.49 nM). These oligonucleotides have the following sequences:

[00744] Oligonucleotide 199:

[00745] 5'- TTATCTTTGTGGGTGACAGGTTTTTCCTGTCACCC-3'-ALEXA FLUOR 647

[00746] Oligonucleotide 192:

[00747] 5'-TTTTTTTGCCCCCAGGGTGACAGGTTTTTCCTGTCACCC-3'-ALEXA FLUOR 647

[00748] The binding of conjugate to the dye-labeled oligonucleotide was measured by detecting the fluorescence intensity at 670 nm (AF647 acceptor wavelength) and 580 nm (Cy3B donor emission wavelength) with 540 nm excitation (FRET) at various oligonucleotide concentrations. The fluorescence intensity (FIG. 7, Y axis) was plotted against the fluorescence wavelength (FIG. 7, X axis). As indicated in FIG. 7 (left panel), the conjugate of Example 4 showed high emission at donor wavelength; in the presence of the acceptor-labeled oligonucleotide, the acceptor fluorescence increased and donor fluorescence decreased. Negative control reactions included the AF-647 labeled oligonucleotide but with no conjugate included, as well as a second control comprising buffer alone. The FRET efficiency was calculated based on the decrease in donor signal after addition of dye-labeled oligonucleotide.

[00749] Example 5: Preparation of a second labeled polymerase conjugate

[00750] 80 μ L of an 111 μ M stock solution of His-tagged polymerase comprising the amino acid sequence of SEQ ID NO: 34 (referred to herein as “HP1-B104 exo-polymerase”) in 10 mM Tris (pH 7.5), 100 mM NaCl, 4 mM DTT, 0.5% v/v Tween-20, 0.1 mM EDTA and 50% v/v glycerol) was buffer exchanged into 1x phosphate buffered saline (1xPBS) pH 7.4 with additional 200 mM NaCl using an NAP-5 column.

[00751] The buffer-exchanged His-tagged polymerase (150 μ L, 18.9 μ M in 1xPBS pH 7.4 and 200 mM NaCl) was mixed with 800 μ L of a buffer comprising 1x PBS pH 7.4 and 200 mM NaCl. The mixture was added into a vial containing 10.8 μ L of 1.3 mM Cy3B NHS ester in DMSO in a 5:1 dye to polymerase molar ratio. The reaction solution was mixed and rotated at 4 °C for 2 hour, and then centrifuged for 5 minutes at 16.8K ref. The supernatant of the conjugation solution was loaded by a syringe onto a Ni-NTA cartridge that was previously washed by ~6 mL water and then by ~6 mL of 100 mM Tris buffer (pH 7.5) with 300 mM NaCl and 1 mM DTT. The column was continuously washed with 100 mM Tris buffer (pH 7.5) with 300 mM NaCl and 1 mM DTT to completely remove the unbound dye. The conjugate retained on the column during the wash was eluted from the cartridge by using 100 mM Tris buffer (pH 7.5) with 0.5 M imidazole, 300 mM NaCl and 1 mM DTT. The solution collected from the cartridge was centrifuged and transferred into a 10K MWCO dialysis cassette. The solution was then dialyzed at 4°C overnight into 50 mM Tris buffer pH7.5 with 150 mM NaCl, 0.2 mM EDTA, 0.1% v/v Tween-20, 5 mM DTT and 50% v/v glycerol. The dye:enzyme stoichiometry of the resulting conjugate preparation was measured using UV absorbance to estimate the concentration of the protein (280 nm) and Cy3B dye (564 nm) and was determined to be about 2 Cy3B dye label per polymerase. This conjugate preparation was assayed to determine concentration, DNA extension activity, and DNA binding by FRET as described in Example 4, above.

[00752] FIG. 6 shows the results of the extension assays to measure primer extension activity of conjugates prepared according to the method of Example 4 (referred to in FIG. 6 as “Cy3B-B104 dy1” and comprising an average of 1 dye per polymerase) and

Example 5 (referred to in FIG. 6 as “Cy3B-B104 dy2” and comprising an average of 2 dyes per polymerase), as well as of a control unconjugated polymerase comprising the amino acid sequence of SEQ ID NO: 34 and further comprising a His-tag and the HP1 linker (referred to in FIG. 6 as “B104”). These results indicate that the negative control reaction wells (no nucleotide) exhibit steady baseline fluorescence level over time compared to conjugate reaction wells or positive control (unconjugated polymerase), which show increasing fluorescence level over time. Based on the fluorescence traces depicted in FIG. 6, the extension activity of the two conjugates was calculated and compared to the extension activity of the positive control (unconjugated polymerase). The results are shown in Table 1, below:

Table 1: Extension activity of labeled polymerase conjugates and unconjugated polymerase

Conjugate	Activity
Cy3B-B104 dy1	0.41 base/sec/conj
Cy3B-B104 dy2	0.42 base/sec/conj
B104 stock	0.34 base/sec/enz

[00753] As indicated in Table 1, above, the two conjugates prepared according to the methods of Examples 4 and 5, respectively, exhibited comparable levels of extension activity as compared to the control (unconjugated) polymerase.

[00754] FIG. 7 depicts the results of the FRET assays to measure DNA binding on conjugates prepared according to the method of Example 4 (referred to in the Figure as “Cy3B-B104 dy1” and comprising an average of 1 dye per polymerase, left panel) and Example 5 (referred to in the Figure as “Cy3B-B104 dy2” and comprising an average of 2 dyes per polymerase, right panel). The binding of conjugate to the dye-labeled oligonucleotide was measured by detecting the fluorescence intensity at 670 nm (AF647 acceptor wavelength) and 580 nm (Cy3B donor emission wavelength) with 540 nm excitation (FRET) at various oligonucleotide concentrations. The fluorescence intensity (FIG. 7, Y axis) was plotted against the fluorescence wavelength (FIG. 7, X axis). As indicated in FIG. 7 (left panel), the conjugate of Example 4 showed high emission at donor wavelength; in the presence of the

acceptor-labeled oligonucleotide, the acceptor fluorescence was increased and donor fluorescence decreased. Negative control reactions included the AF-647 labeled oligonucleotide but with no conjugate included, as well as a second control comprising buffer alone. The FRET efficiency was calculated based on the decrease in donor signal after addition of dye-labeled oligonucleotide according to the following formula:

$$FRET_{eff}(\%) = \frac{I_0 - I}{I_0} \times 100$$

[00755] where:

[00756] I_0 is the conjugate's donor fluorescence intensity at the absence of AF647 labeled oligo199;

[00757] I is the conjugate's donor fluorescence intensity at the presence of AF647 labeled oligo199.

[00758] Using these procedures, the calculated FRET efficiency of each conjugate was approximately 60%. These results indicate that both conjugates comprise donor Cy3B dye linked to the the phi29 polymerase, and that the polymerase of both conjugates retains DNA binding activity with high FRET efficiency to the oligonucleotide acceptor label.

[00759] **Example 6: Single molecule sequencing using labeled polymerases conjugates**

[00760] **PEG-Biotin Surfaces:**

[00761] Glass coverslips surfaces were plasma cleaned and treated with a mixture of poly-ethyleneglycol (PEG) and biotin-PEG to produce a low density biotin surface with a PEG coating to prevent non-specific background of proteins and macromolecules.

[00762] **Fluidic Chamber Assembly:**

[00763] Fluidic cassettes were assembled with glass coverslips to create fluidic chambers capable of containing approximately 20 ul of fluid.

[00764] **Attaching biotinylated DNA to low density PEG-biotin surfaces:**

[00765] Streptavidin protein was diluted to 200 pM in incubation buffer (50 mM NaCl; 50 mM Tris-Cl pH = 7.5; 0.5% BSA). Diluted streptavidin was flowed into fluidic chamber and left to incubate for 10 minutes. Chambers were washed once with 1 ml incubation buffer. Biotinylated-DNA templates were diluted to 200 pM in incubation buffer and allowed to bind for 5 minutes. Surfaces were washed 1 X with 1 ml incubation buffer.

[00766] SA-Polymerase Preparation:

[00767] Labeled polymerase conjugates comprising a Phi-29 polymerase comprising the amino acid sequence of SEQ ID NO: 40 was conjugated to Streptavidin-Cy3B according to the methods of Examples 1. Briefly, dye-labeled streptavidin (consisting of Cy3B dye labels linked to streptavidin according to the method of Example 1 and at a average ratio of about 3.1:1 = dye:streptavidin) was mixed with biotinylated Phi29 polymerase comprising the amino acid sequence of SEQ ID NO: 3 at a 1:1 ratio in 1X PBS. The dye loading distribution (i.e., relative stoichiometry of dye and protein) of the dye-labeled streptavidin preparation was measured using UV absorbance; the results are depicted in FIG. 8.

[00768] SA-Cy3B-bPhi29 binding to Templates:

[00769] SA-Cy3B-b-Phi29 was diluted to 1 nM in binding buffer (150 mM NaCl; 50 mM MOPS pH = 6.8; 0.3% BSA). Surfaces were incubated for 5 minutes with 1 nM SA-Cy3B-b-Phi29. Surfaces were washed with 1 X 1 ml incubation buffer.

[00770] Fluorescence Imaging:

[00771] The microscope body was purchased from Olympus and was outfitted with a TIRF objective lens (100 X; 1.45 NA). The excitation light passes through an excitation filter (EX FT - 543/22), and dichroic mirror (DM - 532) and the sample was epi-illuminated (Coherent) using TIR at approximately 100 W/cm². Upon excitation, resulting epifluorescence emission was passed through an emission filter (EM FT - 540LP) and the resulting emission was split into three paths ("triview" format) using 2 dichroic mirrors and the appropriate bandpass filters for the dye sets of choice. The emission was imaged on a CCD camera. This detection setup is depicted in FIG. 9. Images were collected at a frame rate of approximately 30 ms. Images depict single DNA strands complexed with single SA-Cy3B-bPhi29 conjugates

(donor molecules in this Example) and FRET signals from acceptor species (hexaphosphate 647 and/or 680 nucleotides) bound in the enzyme active (not shown).

[00772] Characterization of Donor Lifetime for SA-Cy3B-Phi29 Conjugates:

[00773] The average donor lifetime for the SA-Cy3B-Phi29 at laser power densities relevant for pattern sequencing experiments was characterized. From an estimated 1000 donors per field of view (1000 donors/FOV) at each of the respective power densities, the donor lifetimes before photobleaching were histogrammed at various power densities (Figure 10 (A)). The average donor lifetime was estimated to be approximately 2 minutes at the power density used for these experiments, 100 W/cm² (FIG. 10(B)).

[00774] In addition, the photon counts to both the 647 and 680 dyes at the relevant excitation powers and the average FRET efficiency for each of these respective dyes were characterized (Figure 11, left). This characterization was performed by trapping the next correct nucleotide in the active site of the enzyme complexed with a DNA strand using calcium. From this experiment, the approximate FRET efficiency for acceptor and donor dye combinations used in FRET based pattern sequencing was deduced (Figure 11, right). The boxed red rectangles in FRET efficiency curves (FIG. 11, right) are the FRET efficiency distributions and means for FRET efficiency, calculated at all power densities.

[00775] Sequencing Reaction:

[00776] Hexa-phosphate dye-labeled nucleotides were diluted to 250 nM in extension buffer. (50 mM MOPS pH = 6.8; 50 mM potassium acetate (pH = 7.0); 0.3% BSA; 5 mM CaCl₂; Katalase 10,772u/ml; Glucose oxidase 0.5mg/ml; Glucose 0.2%). This mixture was flowed into a channel containing SA-Cy3B-bPhi29 bound to DNA template, and images were recorded for approximately 2 minutes at approximately 30 ms frame rates. In one representative example, the DNA template sequence comprised the following sequence (G)₅(A)₈ immediately following the primer annealing site. Using 250 nM hexaphosphate-647-dGTP, 250 nM hexaphosphate-680-dATP and 1 μM cold dTTP, patterns were identified with spectral signatures for 647 dye emission (G signal) preceding spectral signatures for 680 dye emission (A signal) that resulted from fluorescence resonance energy transfer (FRET)

from the Cy3B molecule (donor) linked to the Phi-29 polymerase. Exemplary time traces observed in this assay are depicted in FIG. 12.

[00777] The same sequencing pattern, using the same template and nucleotide combination, was also demonstrated by inverting the color sequencing, such that 250 nM hexaphosphate-680-dGTP and 250 nM hexaphosphate-647-dATP and cold dTTP at 1 μ M were used. In this example, patterns were identified with spectral signatures for 680 dye emission (G signal, long grey spike) preceding spectral signatures for 647 dye (A signal, short grey spikes) emission that resulted from fluorescence resonance energy transfer (FRET) from the Cy3B molecule (donor) linked to the Phi-29 polymerase. Exemplary time traces observed in this assay are depicted in FIG. 13.

[00778] Analysis of Fluorescence Data To Extrapolate Sequence Information

[00779] To convert the observed fluorescence emissions detected during the sequencing reaction into nucleotide sequence information, the raw data comprising a movie of observed emissions was first processed by using a Hidden Markov Model (HMM)-based algorithm to detect and identify FRET events. The subsequent detected FRET events were filtered and filtered sequences were aligned. Each of these two steps, FRET event detection and sequence analysis, are described in more detail below.

[00780] Detection of FRET events

[00781] The analysis underlying FRET event detection is designed to process spatially correlated movie(s) comprising real time sequence fluorescence emission data, and extract time-series of interest from those data. A movie typically contains one or more channels where each channel represents the same spatial location at different wavelengths. The analysis chain begins with the submission of one or more movies to the analysis machine via a comprehensive user interface. The user interface requires the user to input various parameters that describe the movie(s) (e.g. channel regions, dye emission properties, and the like). Once this data is submitted the movie(s) are then processed by the image analysis software where a sliding window of N frames propagates through the movie calculating a temporal local average of the frames within the window. At each position of the window in the movie, the local average image is then further processed and enhanced using well known image processing algorithms and a record of the maximum projection of all the local

average images is recorded to produce a global image of the movie. This global image is the input into a spot identification algorithm which produces a set of spots identified by a unique spot id, its x and y location and its corresponding channel. Each set of spots for a given channel is then registered to the set of spots in every other channel. In this way a set of spot tuples is constructed. If a detected spot in one channel does not have a corresponding detected spot in another channel, then the position of the undetected spot using the transformation between the two channels and the location of the detected spot is inferred. Once a complete set of spot tuples is constructed the movie is iterated over and at each frame the amplitude of each spot is calculated and appended to the appropriate time-series.

[00782] The collection of time-series from a spot tuple consists of time-series from donor and corresponding acceptor channels. This collection is called a Vector Time-Series (VTS). The FRET detection process starts with a data segmentation step using a Markov Chain Monte-Carlo (MCMC) algorithm. Each segment of VTS is modeled by a multivariate Gaussian model, with each of the channel modeled by a mean and a standard deviation. This model establishes a baseline for each channel, from which quantities such as "Donor Down" and "Acceptor Up" can be calculated. A Hidden Markov Model (HMM) is used to model the observed data. The underlying states consist of a null state, a blink state and a number of FRET states (one for each acceptor channel). Each state has its emission probability, which reflects the state's corresponding physical concept. FRET states are characterized by significant "donor down" and "acceptor up" signals. Blink state is characterized by significant "donor down" with no "acceptor up". Null state is characterized by no "donor down" and no "acceptor up". Given the observed VTS signal, the emission matrix, and a state transition probability matrix, the most probable state path can be computed using the Viterbi algorithm. This state path assigns each of the frames to a state. Temporally neighboring FRET frames are grouped into FRET events. For each of the detected FRET events, a list of event features are calculated, including event duration, signal average, signal to noise ratio, FRET efficiency, probability of event, color calling and other features. This list of events and corresponding features are stored in a file.

[00783] The final stage of the automated analysis generates a report summarizing the results in the form of a web page containing summary image, statistics of the spots and FRET detection, together with line intensity plots and base call plots.

[00784] Using the above process, the movie data obtained from the sequencing reactions was analyzed to detect and identify FRET events according to the process described above. The FRET events were then processed to identify sequences as described below.

[00785] Sequence Analysis

[00786] Beginning with the set of detected Förster resonance energy transfer (FRET) events, a data overview was constructed in the form of a color image interpreted as a sequencing plot. To generate the plot, the original FRET event data was pre-processed using a set of filters constructed by *a priori* knowledge of the sequence. For each reaction site (each molecule) an ordered sequence of FRET events was constructed. The base call letters for each FRET event (e.g. "A", "C", "G" or "T") were concatenated to form a sequence ASCII string. The order of letters in the string reflects the temporal relationship of the events. Given that the expected sequence was known *a priori*, a regular expression was then constructed that represented the full or partial expected sequence or sequence pattern. Matching against the regular expression (expected sequence) was then computed for each sequence in the set and the start and stop indices of the match were recorded. A color plot image was then constructed where each row corresponds to a sequence in the set. The plot image was padded to accommodate sequences of different lengths. A color map of $2*N + 1$ colors was constructed, where N denotes the number of possible base calls in each sequence (N=2 for the plot of this Example). N colors were assigned to the base characters that fell within the pattern, N colors were assigned to the base characters that did not fall within the pattern (muted color), and finally a color was assigned to the padding (background) of the image. The rows of the image were then sorted according to the number of base calls in the first part of the sequence pattern. The rows of the image were also aligned such that the start of the expected sequence is in the same column for all rows of the plot.

[00787] Representative results of the sequence analysis are depicted in FIG. 14. Pattern results are represented by the aligning the sequences, where bright yellow

represents the initial onset of the G signal and sequence, and bright pink indicates the onset of the A signal and sequence. Greyed yellow and pink boxes represent outlier signals that were detected using HMM FRET detection which lie outside of the typical sequence onset (HMM detected FRET events shown with gray bars, FIG. 14, top).

[00788] In an exemplary assay that used 250 nM hexaphosphate-647-dGTP and 250 nM hexaphosphate-680-dATP and cold dTTP at 1 μ M, approximately 50% of the total filtered donor spots (single molecule DNA/enzyme complexes) showed the correct pattern for 647 emission signals before 680 emission signals (FIG. 14, bottom).

[00789] As a negative control, 250 nM hexaphosphate-647-dGTP and 250 nM hexaphosphate-680-dATP, cold dTTP at 1 μ M, and cold dGTP at 20 μ M were mixed. Representative results of this control sequence analysis are depicted in FIG. 15. One of the total filtered donor spots (single molecule DNA/enzyme complexes) showed a sequence that resembled the patterns found in the positive lane. (FIG. 15).

[00790] **Example 7: Comparison of photostability of exemplary polymerases and labeled polymerase conjugates**

[00791] The photostability of an exemplary control (unconjugated) polymerase comprising a His-tagged version of Phi-29 polymerase ("HP1; see, e.g., U.S. Provisional Application No. 61/184,770, filed June 5, 2009 for disclosure of HP1 sequence and purification) comprising the amino acid sequence of SEQ ID NO: 14, and an exemplary labeled polymerase conjugate comprising a Phi-29 polymerase including the amino acid sequence of SEQ ID NO: 3 conjugated to a dye label according to the methods of Example 1, were characterized and compared. The photostability was determined by measuring the amount of primer extension observed in each sample prior to and following exposure to excitation radiation at 405 nm. Reactions (100 μ L) containing 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM $MnCl_2$, 0.3% BSA, 10-200 nM polymerase, and 100 nM of 5'-TAMRA-labeled templates were prepared with and without the Oxygen Scavenging System (OSS). The OSS consists of 0.1 mg/ml Glucose Oxidase (Sigma, Catalog # G3660-1CAP), 2 units/ μ L Katalase (Fluka, Catalog # 02071), 2 mM Trolox, and 0.5% glucose (added

just prior to illuminations with 532 laser). Aliquots (4 μ L) were added to a quartz cuvette having a path length of 1.5 mm and a height of 15 mm (Hellma, 105.252-QS) and illuminated with a 532 nm laser for a specified time and at specified power levels. After illumination, samples were removed and placed on ice until extensions were performed at 23⁰C. The extensions were performed by addition of 5 μ M nucleotide hexaphosphates comprising a hexaphosphate moiety linked to the 3' carbon of the sugar moiety, and further comprising a 6-carbon linker attached to the terminal phosphate but without any fluorescent label. Extensions were performed for 30 seconds followed by termination with loading buffer (90% formamide, 10 mM EDTA). Samples were resolved on denaturing polyacrylamide gels (8M urea, 20% polyacrylamide) and exposed to phosphorimager screen. Representative results are provided in FIG. 16. An image of the gel is shown in the bottom left corner of the graph; the polymerase activity was quantified as the % of extended primer (as compared to the total starting amount of primer), as measured by densitometric analysis. Phototoxicity is quantified by measuring the percent decrease in polymerase activity that occurs when samples are illuminated compared to non-illuminated samples.

[00792] FIG. 16 shows the % activity remaining (Y axis) after various durations of exposure (time, X axis) to radiation at 532 nm at 20 W/cm². As shown in FIG. 16, the labeled enzyme conjugate (diamonds) retained 80-90% activity following exposure at 20 W/cm² for up to 10 minutes, a level comparable to the activity retained by the control Phi-29 polymerase (squares).

[00793] Example 8: Nucleotide Incorporation with labeled biomolecule conjugates comprising a mutant B103 polymerase linked to a fluorescent dye label

[00794] In this Example, a mutant B103 polymerase comprising the amino acid sequence of SEQ ID NO: 40, was prepared, biotinylated and labeled as outlined below. This modified B103 polymerase comprises the amino acid sequence of SEQ ID NO: 34 and further includes the mutation H370R as well as a biotinylation site and His tag fused to the N-terminus of the protein. The dye-labeled polymerase conjugate was then used to study nucleotide incorporations in single molecule format.

[00795] Preparation of biotinylated polymerase

[00796] The construct HB B104 (H370R) _pAN6 was transformed and expressed in CVB101 (for in vivo biotinylation) cells. The cells were grown at 30°C to OD 0.6 and induced with 0.5mM IPTG. Upon induction 200 uM D-Biotin was added and cultures were moved to 18°C shaker and grown O/N and harvested the following morning. Cell pellets were resuspended in Buffer B and sonicated to lyse. PEI (0.3%) was added to cell resuspension and incubated on ice for 30 min. Cell resuspension was centrifuged to remove cell debris and DNA. Ammonium sulfate was added to cell lysate at final concentration of 55%. Lysate was centrifuged and pellets containing HB B104 (H370R) were resuspended in Buffer, loaded onto EMD-sulfate column and eluted with linear gradient 10-100% BufferB. Fractions containing HB B104 (H370R) were pooled and loaded onto a His Trap column, eluted with linear gradient from 5-100% Buffer C. Peak fractions were pooled and loaded onto a Heparin column, eluted with a linear gradient from 10-100%B. Fractions were then quantitated and analyzed for polymerase activity.

[00797] Buffer compositions were as follows:

[00798] Buffer A: 30 mM Tris pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EDTA, 8% glycerol

[00799] Buffer B : 30 mM Tris pH 7.5, 1 M NaCl, 2 mM DTT, 0.1 mM EDTA, 8% glycerol

[00800] Buffer C: 30 mM Tris pH 7.5, 100 mM NaCl, 2 mM DTT, 0.5M Imidazole, 8% glycerol

[00801] Preparing NHS-Ester Surfaces:

[00802] Glass coverslips surfaces were plasma cleaned and treated with a mixture of poly-ethyleneglycol (PEG) and NHS-ester to produce a low density NHS-ester surface with a PEG coating to prevent non-specific background of proteins and macromolecules.

[00803] Fluidic Chamber Assembly:

[00804] Fluidic cassettes were assembled with glass coverslips to create fluidic chambers capable of carrying approximately 2 µl of fluid.

[00805] Attaching Amine Terminated Hairpin DNA to Low Density NHS-Ester Surfaces:

Target DNA hairpin sequence:

5'- TTTTTTTTACCCCCGGGTGACAGGTTXTCCTGTCACCC -3'

(SEQ ID NO: 48)

where "X" is an amine group.

[00806] The target DNA was diluted to 500 nM in 1 M NaHCO₃. The diluted target molecules were flowed into the fluidic chamber and incubated for 1 hour. Chambers were washed 1 X with 1 ml deactivating buffer (ethanolamine). Surfaces were washed 1 X with 1 ml incubation buffer (50 mM Tris-Cl, pH = 7.5; 50 mM NaCl; 0.3% BSA).

[00807] SA-Polymerase Conjugate Preparation:

[00808] In this example, dye-labeled Streptavidin was mixed with biotinylated mutant B103 polymerase (b-B103-exo minus) comprising the amino acid sequence of SEQ ID NO: 40 at a 1:1 ratio of SA-protein: b-B103-exo minus in 1X PBS to produce conjugates comprising biotinylated mutant B103 polymerase linked to dye.

[00809] Dye-labeled Streptavidin was purchased from Invitrogen Corp. (Catalog # SA1010). The Cy3-streptavidin was estimated to contain approximately 7-8 Cy3 dyes per streptavidin based on UV absorbance studies (data not shown).

[00810] Briefly, 500 µl of a 3.4 µM solution of Cy3 dye-labeled Streptavidin (Invitrogen, SA1010) was mixed with 25 µl of 200 µM biotin-B104 H370R. Twenty five microliters of 5M NaCl were added to the mixture and it was left at 4 deg C for 1 hour. To remove any free, unconjugated labeled streptavidin, the mixture was diluted with an equal volume of phosphate buffer saline buffer (PBS) and loaded onto a 1 ml HisTrap cartridge (GE Healthcare). Following the loading, the cartridge was washed with PBS until the initially colored eluate from the cartridge became completely colorless. Finally, the bound Cy3 streptavidin – biotin B104 H370R conjugate was eluted off the cartridge with a solution of 500 mM imidazole in PBS buffer containing 200 mM additional NaCl. To the eluted material was added 50 mM biocytin to a final concentration of 5 mM, and the mixture was dialyzed overnight against a solution containing 50% glycerol, 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM DTT.

[00811] SA-Cy3-b-B103 Binding to Templates:

[00812] The conjugates were diluted to 1 nM in binding buffer (50 mM Tris-Cl; pH = 7.5; 0.3% BSA; 100 mM NaCl). The conjugates were flowed into the fluidic chamber which were previously loaded with DNA templates on the surface. Surfaces

were incubated for 5 minutes with conjugates. Surfaces were washed with 1 X 1 ml incubation buffer.

[00813] Fluorescence Imaging:

[00814] The microscope body was purchased from Olympus and was outfitted with a TIRF objective lens (100 X; 1.45 NA). The excitation light passes through an excitation filter (EX FT - 543/22), and dichroic mirror (DM - 532) and the sample was epi-illuminated (Coherent) using TIR at typically 100 W/cm². Upon excitation, the resulting epifluorescence emission passed through an emission filter (EM FT - 540LP) and the resulting emission was split into three paths (tri-view) using 2 dichroic mirrors and the appropriate bandpass filters for the dye sets of choice. Using this filter combination, we were able to spectrally resolve 1 donor dye and 3 acceptor dyes in 3 detection channels.

[00815] In separate experiments, 1 donor dye and 4 different acceptor dyes could be resolved in 4 detection channels. The optical detection scheme was as follows: DC1 = 635, F1 640LP; DC2 = 675, F2 = 688/31; DC3 = 705, F3 = 700 LP. The donor dye used in this case was CY3 and the 4 acceptor dyes are as follows DY634, AF647, AF676, AF700

[00816] The emissions resulting in each experiment were imaged on a CCD camera. Images were collected at a frame rate of approximately 20 ms.

[00817] Three-Color Nucleotide Incorporation Reaction:

[00818] Hexa-phosphate dye-labeled nucleotides were diluted to 200 nM in extension buffer (50 mM MOPS pH = 7.1; 75 mM potassium acetate (pH = 7.0); 0.3% BSA; 1 mM MnCl₂; 300 nM procatuante dioxygenase; 4 mM 3,4 dihydroxyl benzoic acid; 1 mM 2-nitrobenzoic acid; 400 μM 1,2 phenylenediamine; 100 μM ferrocene monocarboxylic acid; 0.02 % cyclooctatetraene; 6 mM TROLOX). Nucleotide mix was flowed into channel with conjugate bound to DNA template and images are recorded for approximately 2 minutes at approximately 20 ms frame rates. In this example, the synthesized strand is expected to have the following sequence: (G)₅T(A)₈. Terminal phosphate-labeled nucleotides and 125 nM cold dC6P were used for the nucleotide incorporation reaction. The labeled nucleotides included 125 nM 647-dT6P, 125 nM 676-dG6P, 125 nM 700-dA6P. The spectral signatures for the ALEXA FLUOR-676 G signal, AF-647 T signal, and AF-700 A signal were

identified that resulted from fluorescence resonance energy transfer (FRET) from the Cy3 donor molecule, and corresponded to the correct insertion sequence pattern.

[00819] Analysis of Three-Color Sequencing Results

[00820] Resulting pattern sequencing data was processed using an alignment algorithm. The alignment algorithm found 100 molecules in the field of view, which demonstrated completion of the full 14-nucleotide sequence ((G)₅T(A)₈, which represented approximately 20% of the total single molecule donor population. The consensus sequence was determined using an HMM alignment algorithm (e.g., see Example 14). By plotting the accuracy definition (measured as a percentage value) against the HMM score (X axis), a linear relationship was detected (data not shown). Various measurements of accuracy can be devised that can be suitable for such analysis. In one exemplary experiment, the accuracy was estimated according to the following equation:

$$\alpha(T, A) = \frac{\beta - \delta - \eta + \lambda}{2\lambda}$$

[00821] The measurement of accuracy in the above equation is intended to provide some measure of similarity between some given template, T, and some alignment, A, of an observed sequence O. It should be noted that alphabet of T, A, and O are identical. The length of T is denoted by λ , the number of deletions in the alignment A by δ , the number of insertions in the alignment by η , and the number of matches in the alignment by β . Equation (1) is normalized by λ such that a an accuracy of 1 indicates a total agreement, and an accuracy of 0 indicates no agreement between T and A. The above definition of accuracy is provided as an example only and is in no way intended to limit the disclosure to any particular theory or definition of accuracy; alternative definitions of accuracy are also possible and it may be suitable to use such alternative definitions in some contexts.

[00822] The accuracy in this system using an HMM alignment threshold of 0 was estimated to be approximately 80% (data not shown).

[00823] Four-Color Nucleotide Incorporation Reaction:

[00824] Oligonucleotides

[00825] 401 template molecule:

[00826] TTTTCCCCGACGATGCCTCCCC g ACA Cgg Agg TTC TAT CAT CgT
CAT CgT CAT CgT CAT Cg-Biotin TEG-T-3 (SEQ ID NO: 49)

[00827] Primer for 401 template:

[00828] 5' TGA TAG AAC CTC CGT GTC 3' (SEQ ID NO: 50)

[00829] In this example, the synthesized strand is expected to have the following sequence: GGGGAGGCATCGTCCGGAAAA (SEQ ID NO: 51)

[00830] Nucleotide Incorporation Reaction:

[00831] Hexa-phosphate dye-labeled nucleotides were diluted to 200 nM in extension buffer (50 mM MOPS pH = 7.1; 75 mM potassium acetate (pH = 7.0); 0.3% BSA; 1 mM MnCl₂; 300 nM procuante dioxygenase; 4 mM 3,4 dihydroxyl benzoic acid; 1 mM 2-nitrobenzoic acid; 400 μM 1,2 phenylenediamine; 100 μM ferrocene monocarboxylic acid; 0.02 % cyclooctatetraene; 6 mM TROLOX). Nucleotide mix was flowed into channel with SA-Cy3-b-B103 bound to DNA template and images are recorded for approximately 2 minutes at approximately 20 ms frame rates.

[00832] The terminal phosphate-labeled nucleotides used for the nucleotide incorporation reaction included 125 nM DY634-dA6P, 125 nM 647-dT6P, 125 nM 676-dG6P, 125 nM 700-dC6P. The spectral signatures for the DY-634 A signal, and the ALEXA FLUOR G, T and C signals (AF-676 G signal, AF-647 T signal, and AF-700 C signal) were identified that resulted from fluorescence resonance energy transfer (FRET) from the Cy3 donor molecule, and corresponded to the correct insertion sequence pattern. 4-color sequence alignment was obtained by visual inspection.

[00833] The observed FRET event durations for various SA-Cy3-b-B103 conjugates, the event count distributions, and the observed extension speeds of various SA-Cy3-b-B103 conjugates were calculated.

[00834] **Example 9: Comparing the primer extension activities of conjugated and unconjugated polymerases**

[00835] This assay describes how to measure and compare the primer extension activity of a labeled polymerase conjugate comprising multiple polymerases per conjugate, with the primer extension activity of unconjugated (free) enzyme. Primer extension activity is quantified by monitoring the fluorescence intensity change over

time during extension of a fluorescein-labeled hairpin oligonucleotide, comprising the following nucleotide sequence. The fluorescence intensity correlates with the level of primer extension activity in the sample.

[00836] Step 1: Measure the primer extension activities of the conjugate and the free (unconjugated) enzyme

[00837] Conjugate primer extension activity is measured by monitoring the fluorescence intensity change over time during extension of a fluorescein-labeled hairpin oligonucleotide, oligo 221 comprising the nucleotide sequence of SEQ ID NO: 43, below:

(5'-TTTTTTTGCAGGTGACAGGTTTTCTGTCA-CC(fluorescein-T)GC-3')
(SEQ ID NO: 43)

[00838] The extension reactions are performed in 1X extension buffer (50 mM Tris buffer pH 7.5, 50 mM NaCl, 10 mM MgCl₂ and 0.5 mM MnCl₂). To reaction wells that contain 100 µL of 150 nM of a fluorescein-labeled hairpin oligonucleotide, oligo221 (SEQ ID NO: 43, above) and 10 nM of polymerase (or conjugated polymerase) in extension buffer, 2 µL of 1 mM dATP (final concentration: 20 µM) is added to initiate the enzymatic reaction and the fluorescence intensity in each well is recorded at 525 nm fluorescence with 490 nm excitation for every 20 seconds for the next 10 minutes. Control reaction wells include the same components without any addition of dATP. The fluorescence intensity at 525 nm (as measured in arbitrary fluorescence units, RFU, y axis) is plotted against time (seconds, X axis) for each sample, as well as the control wells (no nucleotide). The fluorescence time course data from each well is used to calculate the primer extension activity of each sample.

[00839] For conjugate activity (base/sec/conj), the activity is calculated according to the following equations:

$$Activity(base/sec/enz) = \frac{\Delta RFU_{sample - per_sec}}{\Delta RFU_{max - per_nMsubs}} \times \frac{1}{10nM} \times 7(base)$$

$$\text{and } \Delta RFU_{max - per_nMsubs} = \frac{RFU_{max} - RFU_{min}}{substr_conc.(nM)}$$

[00840] Where: RFU_{max} is the average maximal RFU in the reference polymerase reaction wells; RFU_{min} is the average minimal RFU in the reference polymerase

control wells; Substr_conc. (nM) is the oligo 221 concentration in assay, which is 150 nM; and:

$$\Delta RFU_{sample - per - sec} = \frac{RFU_t - RFU_0}{t(sec)}$$

[00841] Where: t (sec) is the time period where the fluorescence intensity increases in the reference enzyme reaction well linearly from the start; RFU_t is the average RFU of the reference enzyme extension wells at t second point; and RFU₀ is the average RFU of the reference enzyme extension wells at the start point.

[00842] For activity of free enzyme (base/sec/enzyme), the activity is calculated according to the following equations:

$$Free - enzyme - activity(base/sec/enz) = \frac{\Delta RFU_{sample - per - sec}}{\Delta RFU_{max - per - nMsubs}} \times \frac{1}{50nM} \times 7(base)$$

$$[00843] \text{ and } \Delta RFU_{max - per - nMsubs} = \frac{RFU_{max} - RFU_{min}}{substr_conc.(nM)}$$

[00844] Where: RFU_{max} is the average maximal RFU in the reference polymerase reaction wells; RFU_{min} is the average minimal RFU in the reference polymerase control wells; and Substr_conc. (nM) is the oligo 221 concentration in assay, which is 150 nM; and

$$[00845] \quad \Delta RFU_{sample - per - sec} = \frac{RFU_t - RFU_0}{t(sec)}$$

[00846] Where: t (sec) is the time period where the fluorescence intensity increases in

[00847] the reference enzyme reaction well linearly from the start; RFU_t is the average RFU of the reference enzyme extension wells at t second point.; RFU₀ is the average RFU of the reference enzyme extension wells at the start point.

[00848] Step 2: Measure the number of active enzyme per conjugate

[00849] This procedure describes a method to measure the active number of polymerase per conjugate based on the active polymerase binding to fluorescein-labeled template using FP as the readout. In this assay, the mP values of a conjugate

at several concentrations are measured and a standard curve is also generated for the mP values at known concentrations of free polymerase. By fitting the mP values into the standard curve, the number of polymerase per conjugate can be calculated

[00850] Extension buffer (50 mM Tris pH7.5 with 50mM NaCl, 10mM MgCl₂ and 0.5mM MnCl₂) is added into the first two rows of a 96-well microtiter plate for 50 µL per well. To the first well of the above each row, 50 µL of 4000 nM free polymerase (polymerase used in the tested conjugate) in extension buffer is added, mixed and transferred 50 µL per well into the second well of the above each row. A 2-fold dilution (concentration from 4000 nM to 1.95 nM) of the free polymerase is then prepared in the above two rows by sub-sequentially transferring and mixing as described. 50 µL of solution is removed from the last well of each row to make 50 µL volume for each well. These two rows are served as the free polymerase standard wells.

[00851] For the wells containing conjugate, 50 µL of conjugate is added to each well with various concentrations (e.g. 20 nM, 40 nM, 60 nM, 80 nM) that are prepared by diluting conjugate into the extension buffer.

[00852] To all wells including either free enzyme or or conjugated enzyme, 50 µL of 300 nM oligo221 in 1X extension buffer is added. The plate is then mixed and the mP value of each well is measured using a plate reader.

[00853] To calculate the active number of polymerase per conjugate, the standard curve is fitted into a non-linear regression equation:

$$Y = b + \frac{a - b}{1 + 10^{(\log EC50 - X) * c}}$$

[00854] Where Y is the mP value;

[00855] X is the log[phi29(nM)];

[00856] a is the top value of the standard curve;

[00857] b is the bottom value of the standard curve;

[00858] c is the slope of the curve

[00859] EC50 is the concentration that gives 50% of the total response.

[00860] Based on the standard curve fitting results, the active polymerase concentration at certain conjugate concentration can be calculated by inputting the mP value at particular conjugate concentration into the equation. The actual number of active polymerase per conjugate is then determined by dividing the calculated active polymerase concentration by the corresponding conjugate concentration.

[00861] Step 3: Calculate the “Ratio of Activity for Conjugated-enzyme to Free-enzyme”

[00862] Based on the results of the measurements for “conjugate_activity”, “Free-enzyme_activity” and the “Nn active polymerase per conjugate”, the ratio of activity of conjugated enzyme to free enzyme (“Ratio of Activity for Conj-enzyme to Free-enzyme”) can be calculated as follows:

$$\begin{aligned} \text{Ratio_Activity_of_Conj-Enzyme-to-FreeEnzyme} &= \\ &= \frac{\text{Conjugate_activity}}{\text{Nn_active-Pol-per-Conjugate}} \times \frac{1}{\text{Free-polymerase_activity}} \end{aligned}$$

[00863] **Example 10: Measurement of t_{-1} and t_{pol} values of modified Phi-29 and B103 polymerases**

[00864] In this example, the t_{-1} and t_{pol} values of a Phi-29 polymerase comprising the amino acid sequence of SEQ ID NO: 3 and a B103 polymerase comprising the amino acid sequence of SEQ ID NO: 34 (referred to as “mB103” in the table below) and including amino acid substitutions at various positions were measured using a stopped-flow procedure. The stopped-flow techniques for measuring t_{pol} ($1/k_{pol}$) followed the techniques described by MP Roettger (2008 Biochemistry 47:9718–9727; M. Bakhtina 2009 Biochemistry 48:3197–320).

[00865] Stopped-Flow Measurements of t_{pol}

Template C sequence:

5'-CGTTAACCGCCCGCTCCTTTGCAAC-3' (SEQ ID NO: 44)

Primer sequence:

5'-GTTGCAAAGGAGCGGGCG-3' (SEQ ID NO: 45)

[00866] The template sequence (SEQ ID NO: 44) further included an Alexa Fluor 546 dye moiety bonded to the 5' position of the template.

[00867] The kinetics of nucleotide incorporation by each polymerase was measured in an Applied Photophysics SX20 stopped-flow spectrometer by monitoring changes in fluorescence from the dye-labeled primer-template duplex complexed to enzyme, following the mixing of the enzyme-DNA complex with dye-labeled nucleotides. These dye-labeled nucleotides comprise terminal-phosphate-labeled nucleotides having an alkyl linker with a functional amine group attached to the dye, and have the general structure shown in FIG. 21. This structure includes a sugar bonded to a hexaphosphate chain at the 5' carbon position, and to a nucleotide base (denoted as "N"). The terminal phosphate group of the hexaphosphate is linked to a 6-carbon linker, and the other end of the 6-carbon linker is attached to a dye moiety (denoted as "dye"), typically through an amide bond. In this example, the particular dye-labeled nucleotide added was a labeled nucleotide hexaphosphate comprising a guanine base at the N (base) position and an Alexa Fluor 647 (AF647) at the dye position, and is referred to herein as "AF647-C6-dG6P".

[00868] The primer and template were annealed to form a dye-labeled primer-template duplex using standard methods. This duplex was preincubated with polymerase. The mixture included 330 nM recombinant DNA polymerase, 100 nM template/primer duplex in buffer ("reaction buffer") comprising 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM DTT, 0.2% BSA, and 2 mM MnCl₂. The dye-labeled nucleotide AF647-C6-dG6P was then added to a final concentration of 7 μM, and the resulting fluorescence was monitored over time.

[00869] The averaged (5 traces) stopped-flow fluorescence traces (> 1.5 ms) were fitted with a double exponential equation (1) to extrapolate the rates of the nucleotide binding and product release,

$$\text{Fluorescence} = A_1 * e^{-k_1 * t} + A_2 * e^{-k_{\text{pol}} * t} + C \quad (\text{equation 1})$$

where A_1 and A_2 represent corresponding fluorescence amplitudes, C is an offset constant, and k_1 and k_{pol} are the observed rate constants for the fast and slow phases of the fluorescence transition, respectively.

[00870] Stopped-Flow Measurements of t_{-1}

[00871] The stopped-flow techniques for measuring t_{-1} ($1/k_{-1}$) followed the techniques described by M. Bakhtina (2009 Biochemistry 48:3197–3208).

Template C sequence:

5'- CAGTAACCGG AGT TGG TTG GAC GGC TGC GAG GC -3' (SEQ ID NO: 46)

Dideoxy-primer sequence:

5'- GCC TCG CAG CCG TCC AAC CAA CTC ddC -3' (SEQ ID NO: 47)

[00872] The rate of the nucleotide dissociation (k_{-1}) from the ternary complex of [enzyme•DNA•nucleotide] was measured in an Applied Photophysics SX20 stopped-flow spectrometer by monitoring changes in fluorescence from in fluorescence from a duplex Alexa fluor 546 dye-labeled-DNA template following the mixing of the [enzyme•DNA•labeled nucleotide] ternary complex with 50 μ M cognate non-labeled deoxynucleoside triphosphate in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4 mM DTT, 0.2% BSA, and 2 mM $MnCl_2$.

[00873] The ternary complexes were prepared using: 330 nM polymerase, 100 nM template/primer duplex, and 7 μ M terminal phosphate-labeled nucleotides (AF647-C6-dG6P).

[00874] The averaged stopped-flow fluorescence traces (>1.5 msec) were fitted with a single exponential equation (2) to extrapolate the rate of the nucleotide dissociation (k_{-1}) from the [enzyme•DNA•nucleotide] ternary complex.

$$\text{Fluorescence} = A_1 * e^{-k_{-1} * t} + C \quad (\text{equation 2})$$

where A_1 represents the corresponding fluorescence amplitude, C is an offset constant, and k_{-1} and the observed rate constants for the fluorescence transition.

[00875] Some representative results of the stopped flow data are shown below.

[00876] Some representative results of the stopped flow data are shown in the Table below:

[00877] Table: Summary of t_{pol} and t_{-1} measurements for various exemplary modified Phi-29 and B103 polymerases

Protein	t_{pol}	t_{-1}
mB103 (SEQ ID: 8)	14	16
mB103 + H370R	17	43
mB103 + H370Y	15	12
mB103 + E371R	11	17
mB103 + E371Y	11	7
K372R	14	12
K380R	783	17
mB103 + D507G	11	13
mB103 + D507H	7	16
mB103 + K509Y	10	20
Phi-29 (exo-)	11	27
Phi-29 (exo-)+T373R	15	81
Phi-29 (exo-)+T373Y	14	45

CLAIMS

What is claimed is:

1. A labeled polymerase conjugate, comprising:
a polymerase linked to at least three labels to form a labeled polymerase conjugate,
wherein the conjugate has polymerase activity.
2. The conjugate of claim 1, wherein the conjugate comprises three, four, five, six, seven or more labels.
3. The conjugate of claim 2, wherein the polymerase activity is at least about 80% relative to the polymerase activity of the unconjugated polymerase.
4. The conjugate of claim 2, wherein the at least three labels are linked to an attachment site on the polymerase.
5. The conjugate of claim 2, wherein the at least three labels are linked to different attachment sites on the polymerase.
6. The conjugate of claim 2, wherein the polymerase further comprises a modification enzyme recognition sequence.
7. The conjugate of claim 6, wherein the modification enzyme recognition sequence is a biotin ligase modification site.
8. A method for creating a labeled polymerase conjugate, comprising:
linking a polymerase to a plurality of labels to form a labeled polymerase conjugate having polymerase activity,
where the plurality of labels are linked to the polymerase.

9. The method of claim 8, wherein the linking further comprises linking the polymerase to at least three labels.
10. The method of claim 8, wherein the linking further comprises linking the plurality of labels to an attachment site on the polymerase.
11. The method of claim 8, wherein the linking further comprises linking the plurality of labels to independent attachment sites on the polymerase.
12. The method of claim 8, wherein the linking further comprises linking a polymerase including a modification enzyme recognition sequence to the plurality of labels.
13. The method of claim 12, wherein the modification enzyme recognition sequence is a biotin ligase modification site, and further comprising linking a biotin moiety to the polymerase to produce a biotinylated polymerase.
14. The method of claim 13, further comprising contacting the biotinylated polymerase with an avidin moiety linked to the plurality of labels under conditions where the avidin moiety binds to the biotin moiety.
15. A labeled polymerase conjugate, comprising:
 - a polymerase linked to a plurality of labels to form a labeled polymerase conjugate,
 - where the conjugate has polymerase activity, and
 - where at least one label undergoes energy transfer with a labeled nucleotide bound to an active site of the polymerase.
16. The conjugate of claim 15, where the labeled nucleotide comprises a polyphosphate and where the nucleotide label is linked to the terminal phosphate of the polyphosphate.

17. The conjugate of claim 15, wherein the energy transfer produces a signal in a single molecule reaction.
18. The conjugate of claim 15, wherein the plurality of labels are connected to the polymerase at an attachment site on the polymerase.
19. The conjugate of claim 15, wherein the plurality of labels are connected to the polymerase at a plurality of attachment sites on the polymerase.
20. The conjugate of claim 15, wherein the plurality of labels includes three or more labels.
21. The conjugate of claim 20, wherein the polymerase comprises a modification enzyme recognition sequence.
22. A method for generating a signal, comprising:
contacting a labeled polymerase conjugate including a polymerase linked to a label with a labeled nucleotide under conditions where the polymerase catalyzes the incorporation of the labeled nucleotide into a nucleic acid, and the label emits, or induces the emission of, a signal indicative of such nucleotide incorporation.
23. The method of claim 22, wherein the labeled polymerase conjugate further comprises at least three labels.
24. The method of claim 22, wherein the plurality of labels are attached to an attachment site on the polymerase.
25. The method of claim 23, wherein the plurality of labels are attached to independent attachment sites on the polymerase.
26. The method of claim 23, wherein the polymerase further comprises a modification enzyme recognition sequence.

27. The method of claim 26, wherein the modification enzyme recognition sequence further comprises a biotinylation site, and the polymerase comprises a biotin moiety.

28. The method of claim 22, further comprising detecting the signal.

29. The method of claim 28, further comprising analyzing the signal to identify the nucleotide that is incorporated.

30. A labeled polymerase conjugate, comprising: a polymerase linked to one or more labels, where the conjugate has polymerase activity and emits upon continuous excitation a total photon count of at least 10^2 photons before irreversibly photobleaching.

31. The conjugate of claim 30, wherein the polymerase is linked to at least three labels.

32. The conjugate of claim 31, wherein the conjugate emits a total photon count of at least 10^8 photons as measured using a test detection system.

33. A labeled polymerase conjugate, comprising:
a first member of a binding pair linked to a polymerase; and
a second member of the binding pair linked to at least one label,
where the first and the second member of the binding pair are linked to each other to form a labeled polymerase conjugate having polymerase activity.

34. The conjugate of claim 33, wherein the first member comprises a biotin moiety and the second member comprises an avidin moiety.

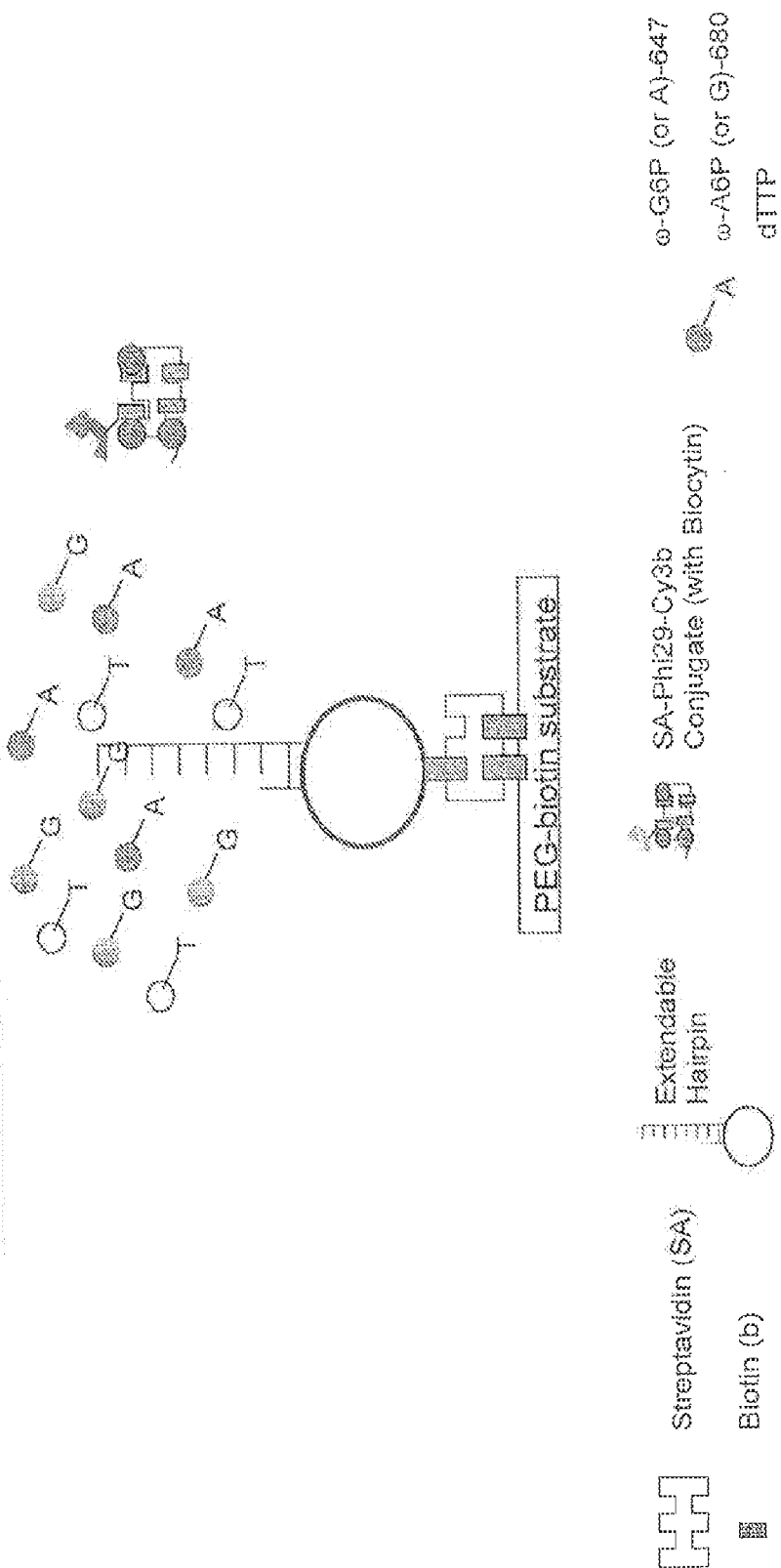
35. The conjugate of claim 33, wherein the second member is linked to at least three labels.

36. The conjugate of claim 33, wherein the at least one label generates a signal.

37. The conjugate of claim 33, wherein the at least one label is positioned to undergo FRET with a labeled nucleotide bound to the nucleotide binding site of the polymerase.


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Insertion Sequence: GGGGG T AAAAAAA



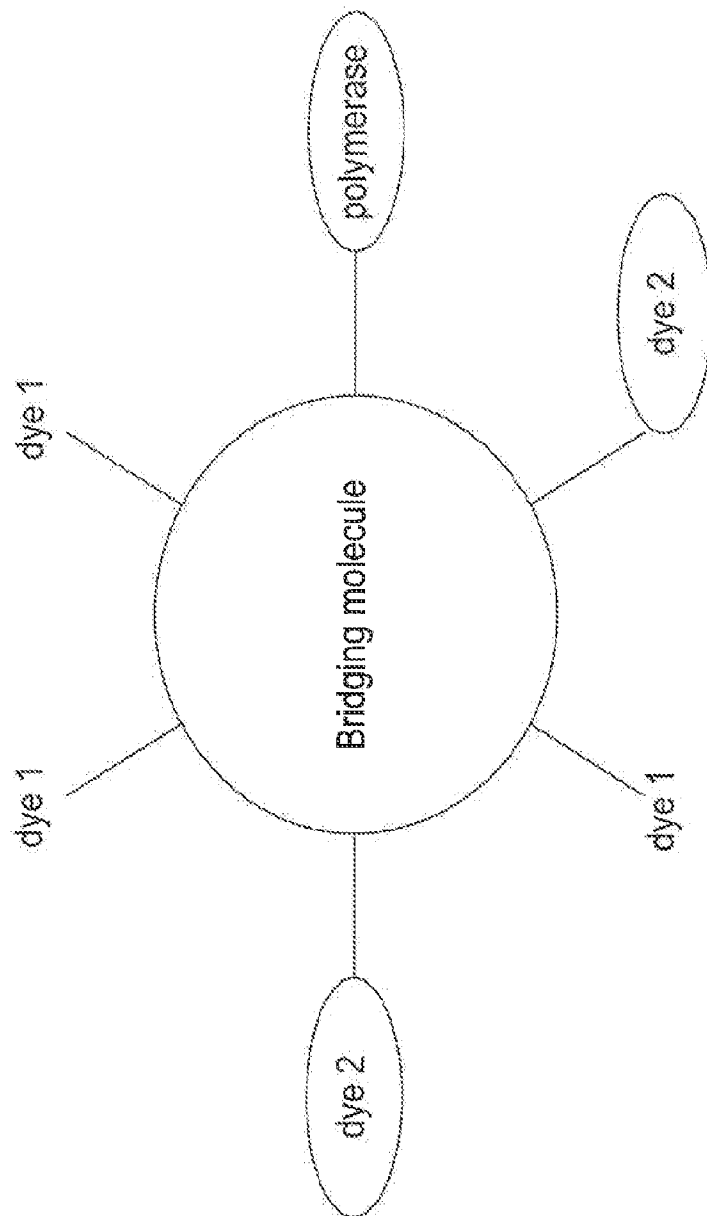


FIG. 2

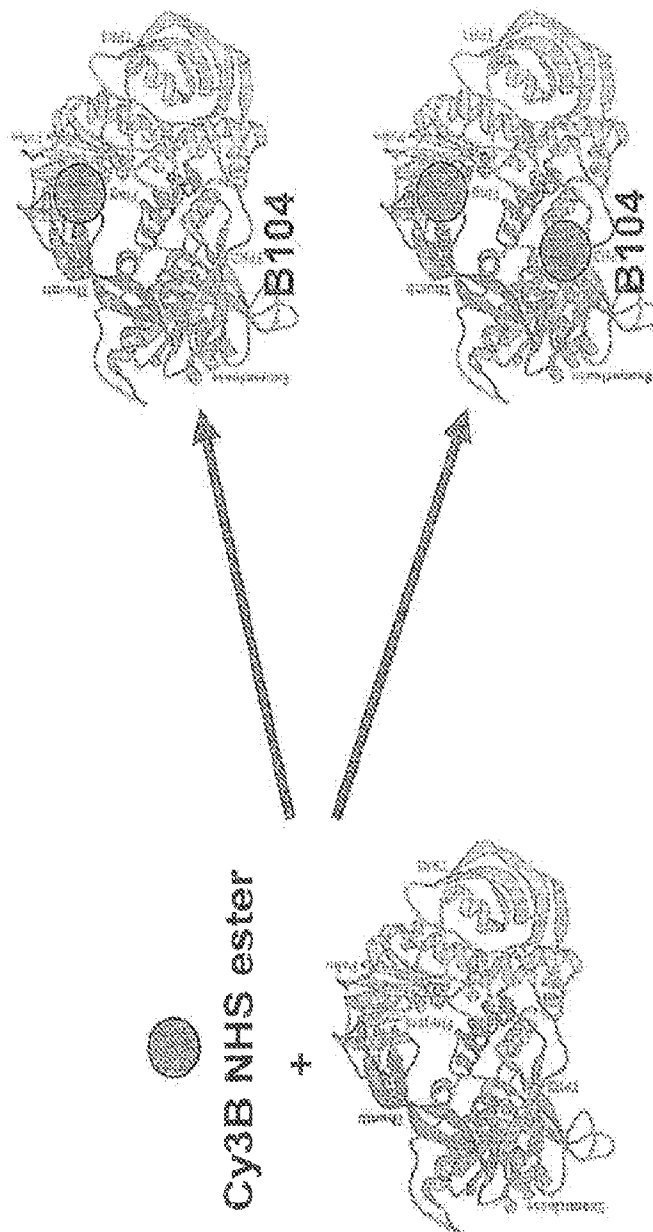


FIG. 3

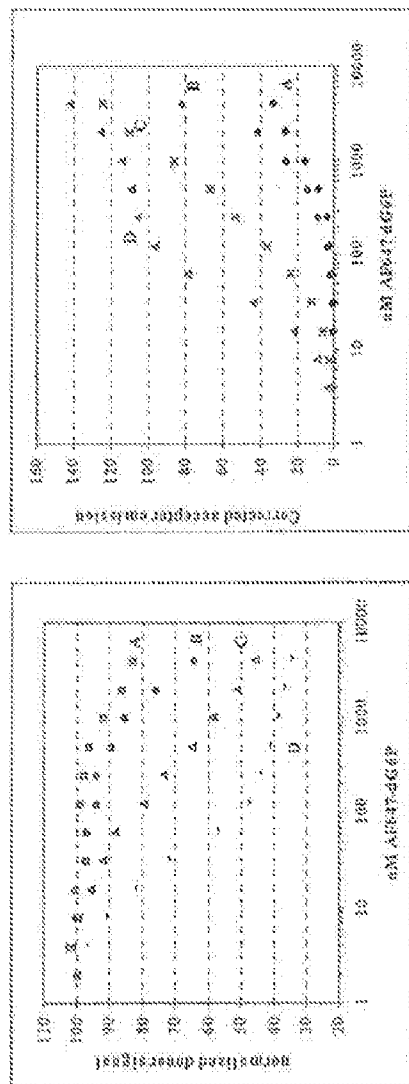


FIG. 4

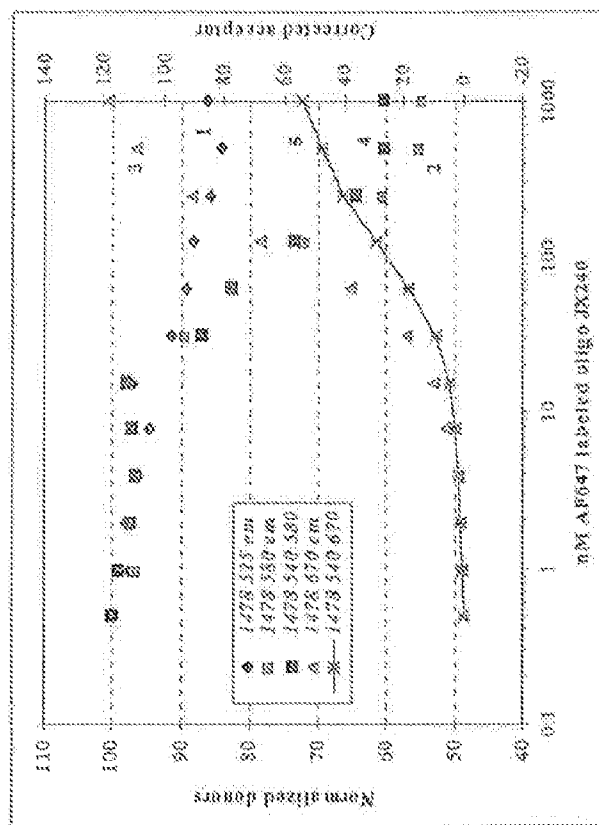


FIG. 5

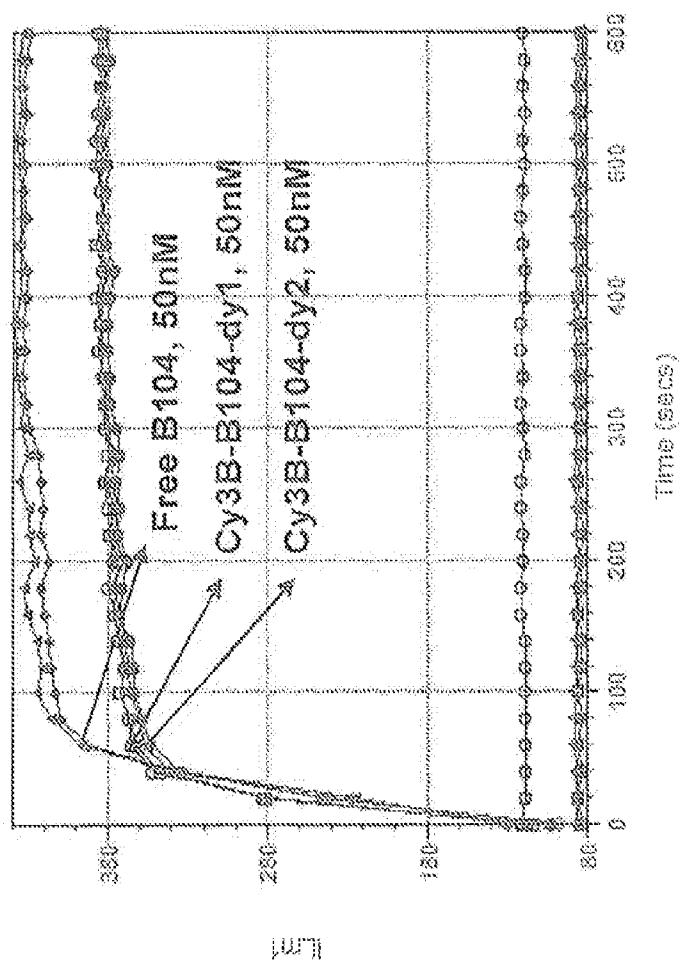


FIG. 6

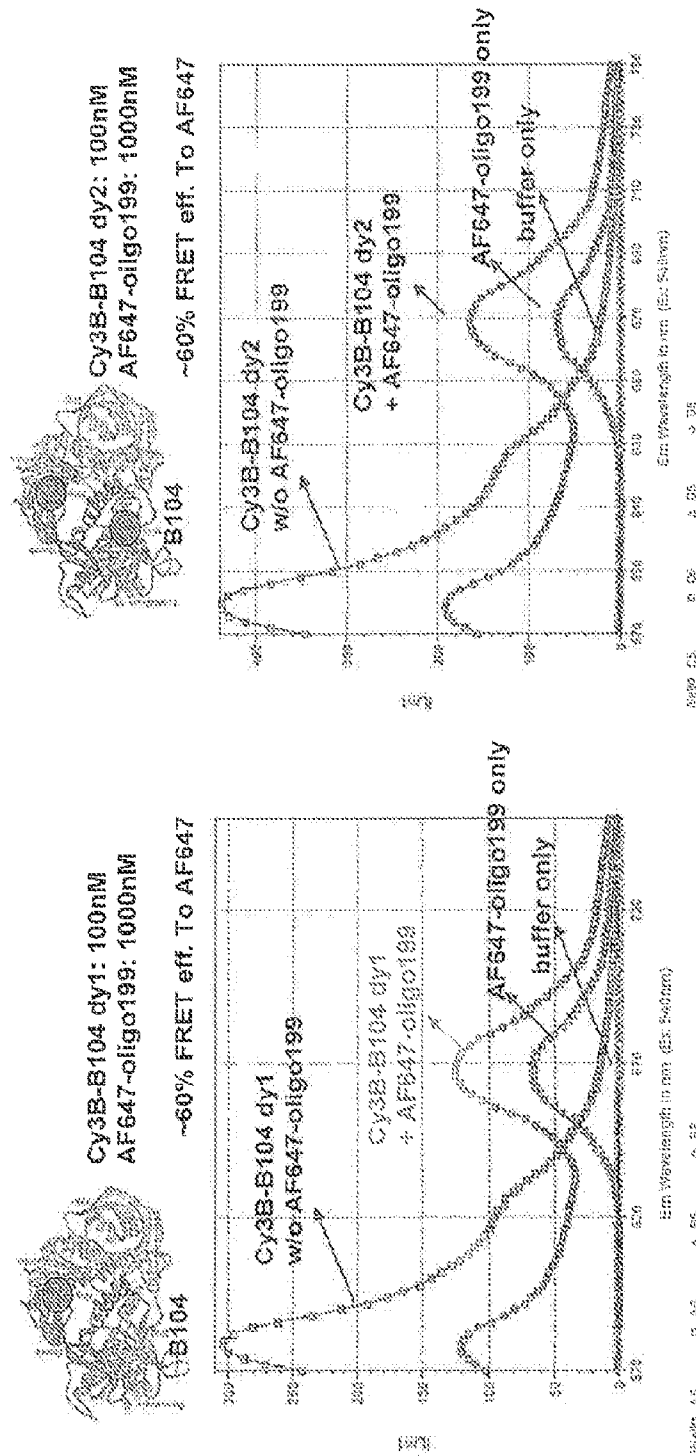


FIG. 7

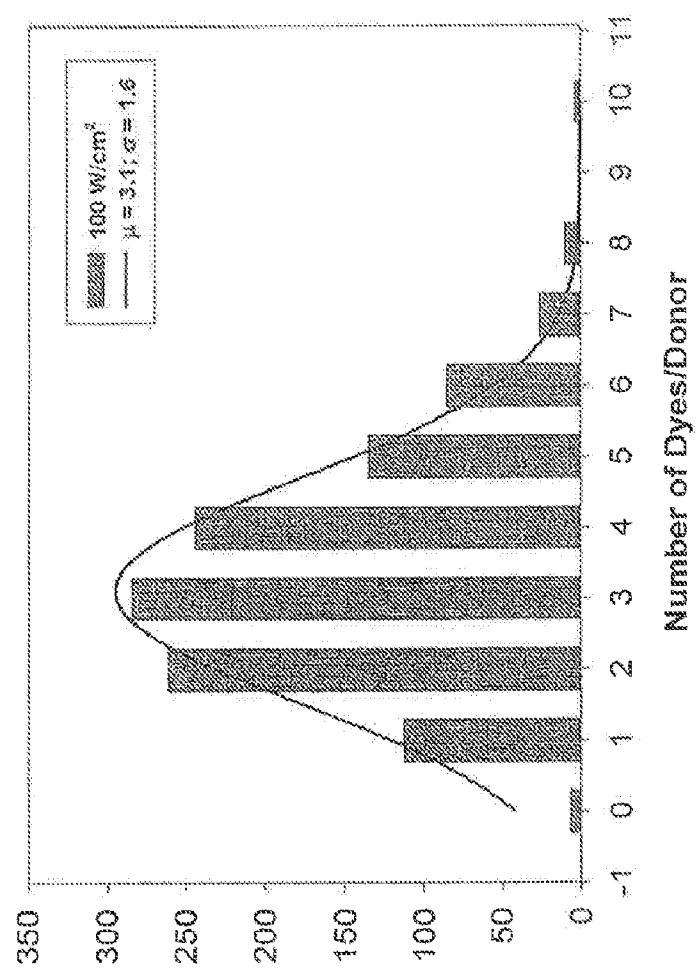


FIG. 8

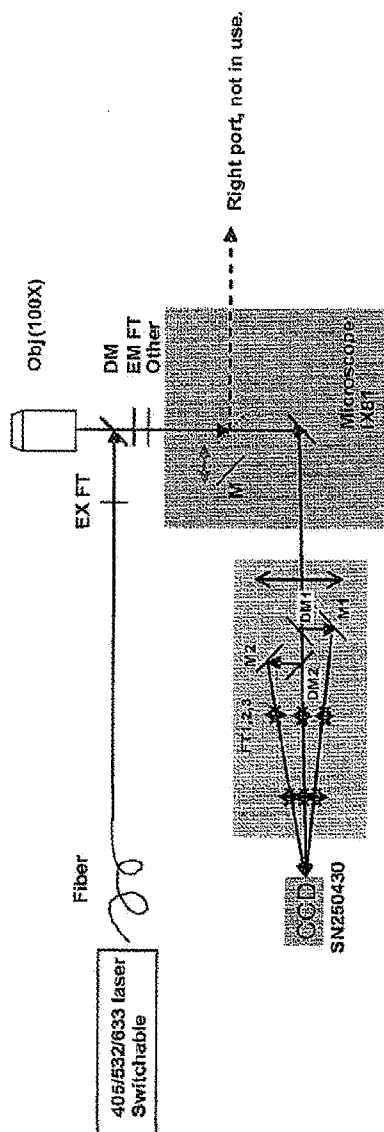
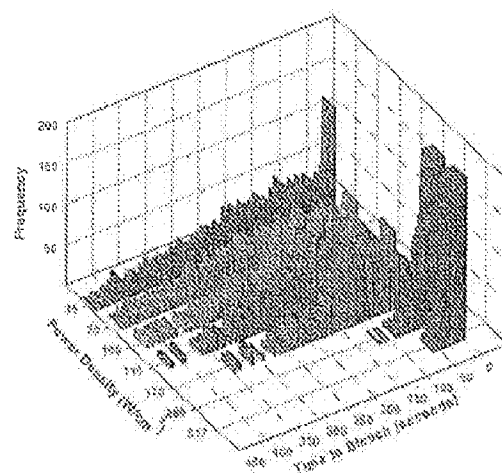


FIG. 9

**FIG. 10(A)**

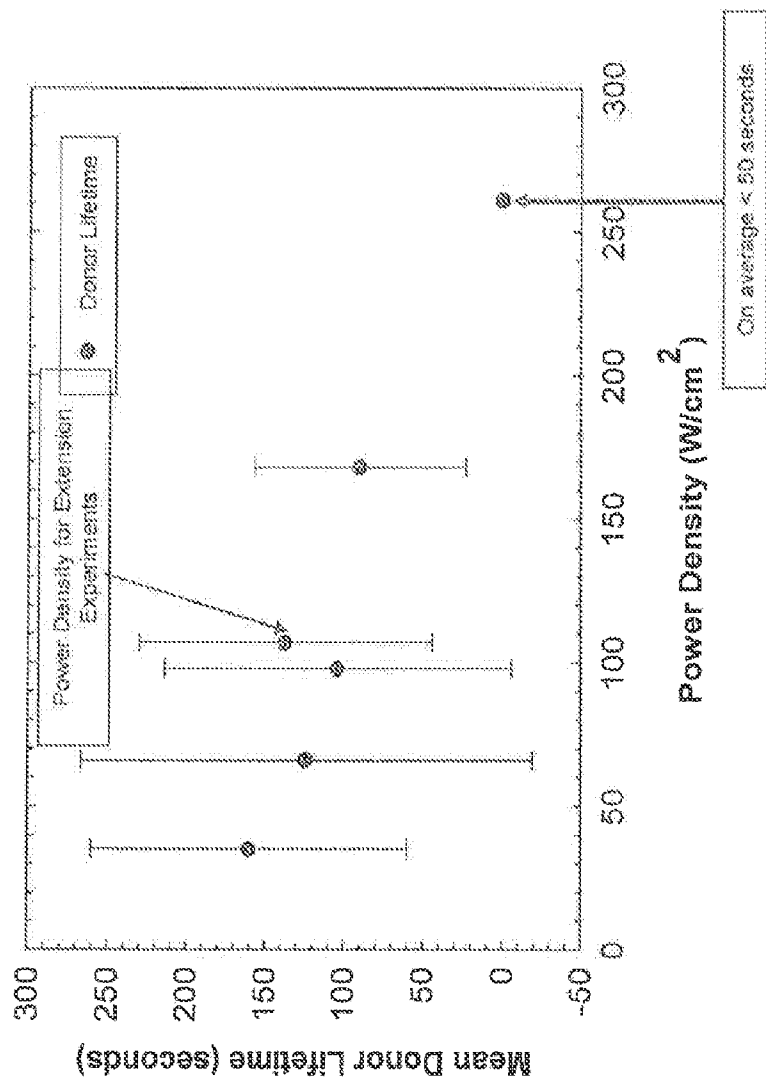
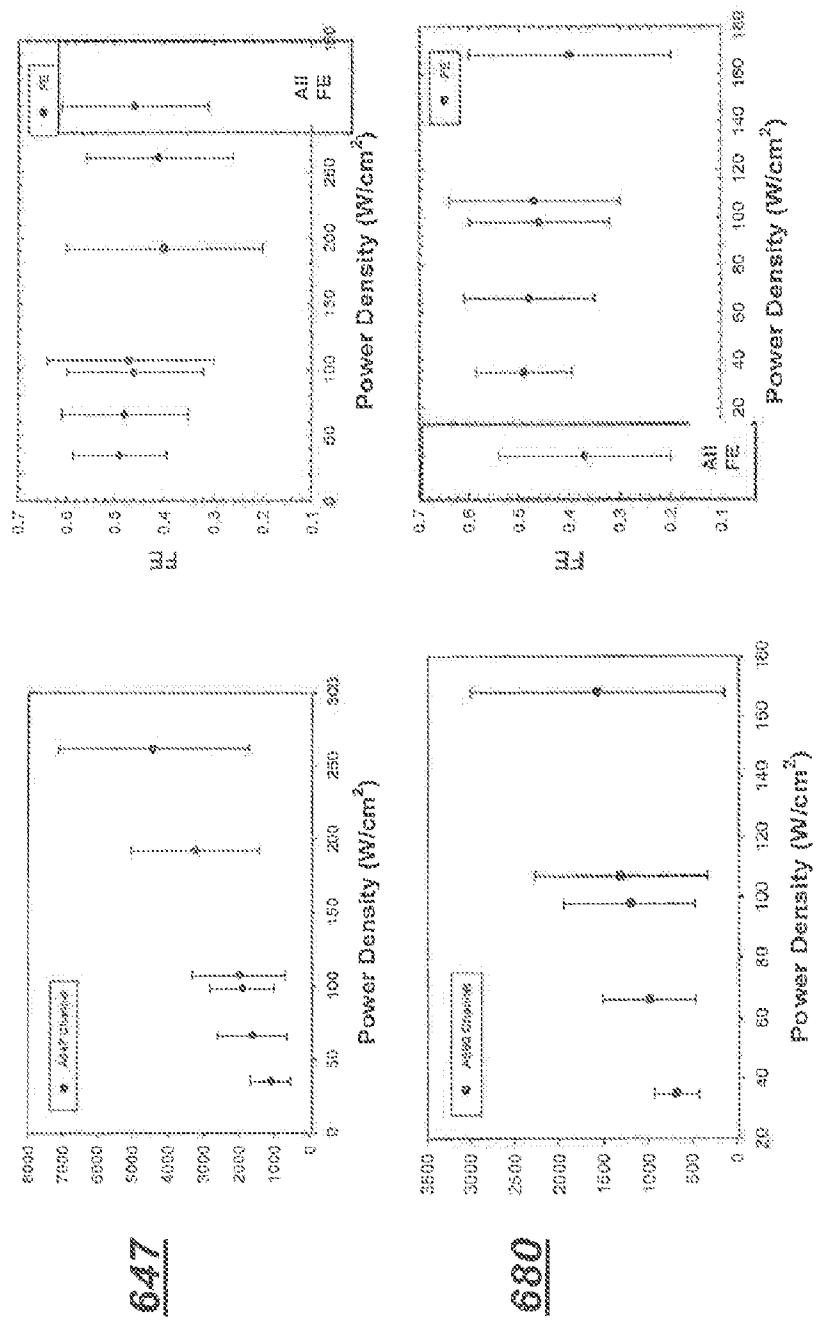


FIG. 10 (B)



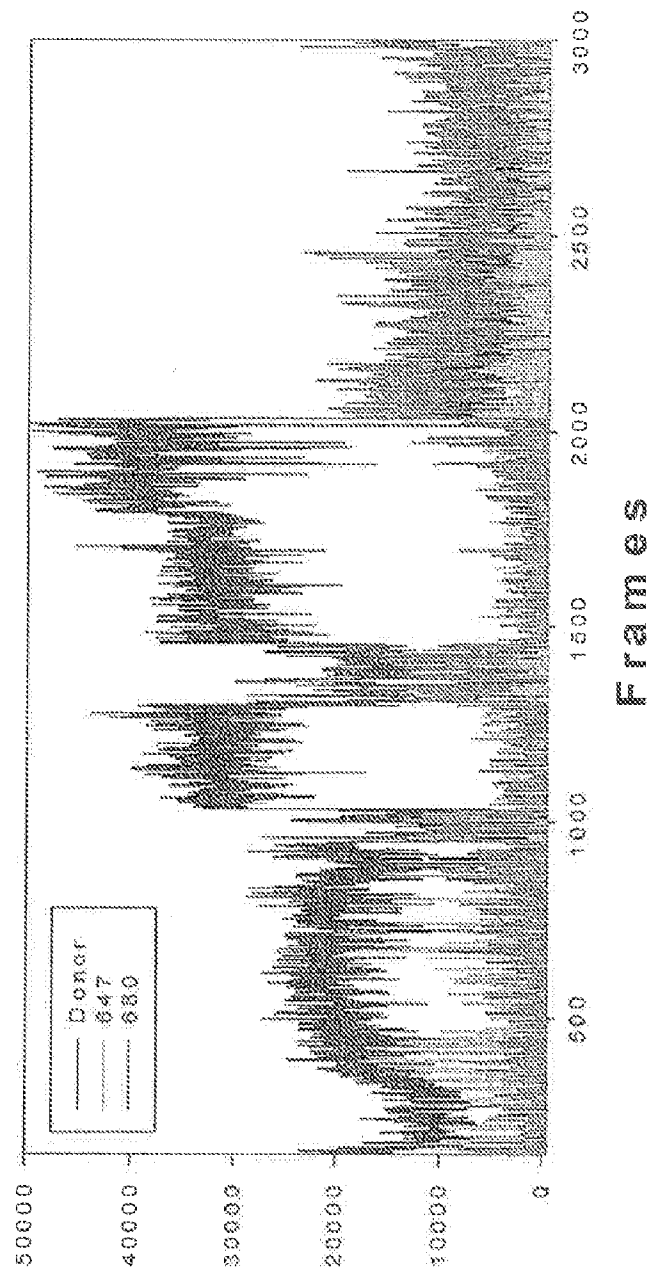


FIG. 12

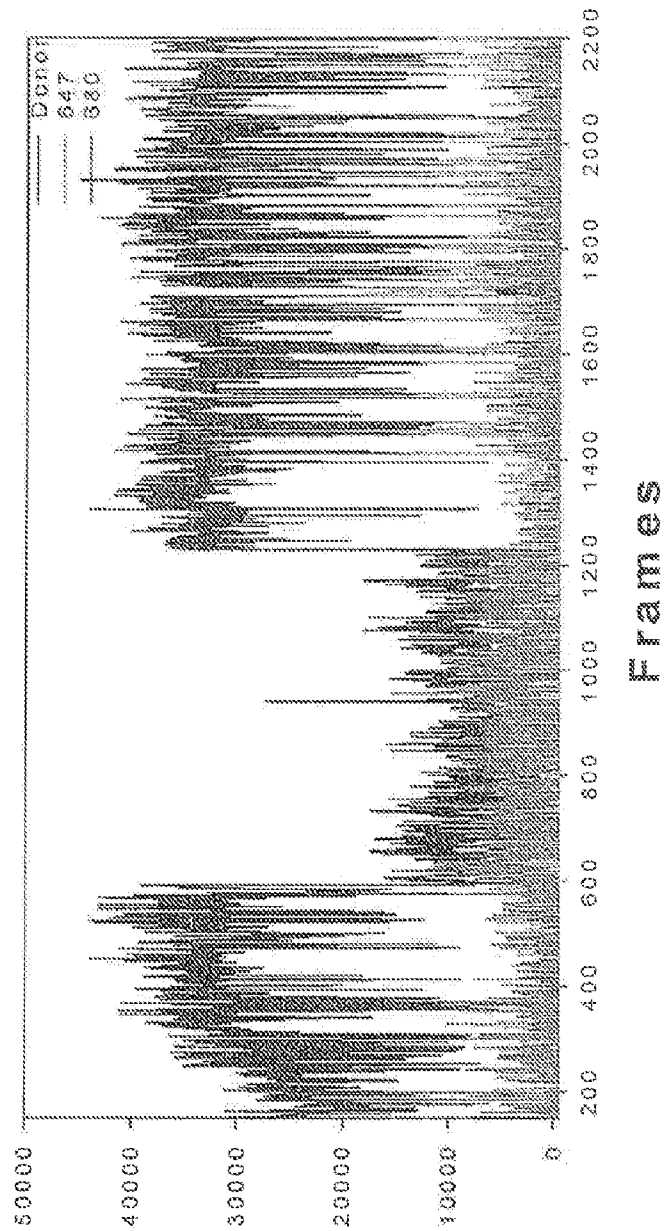


FIG. 13

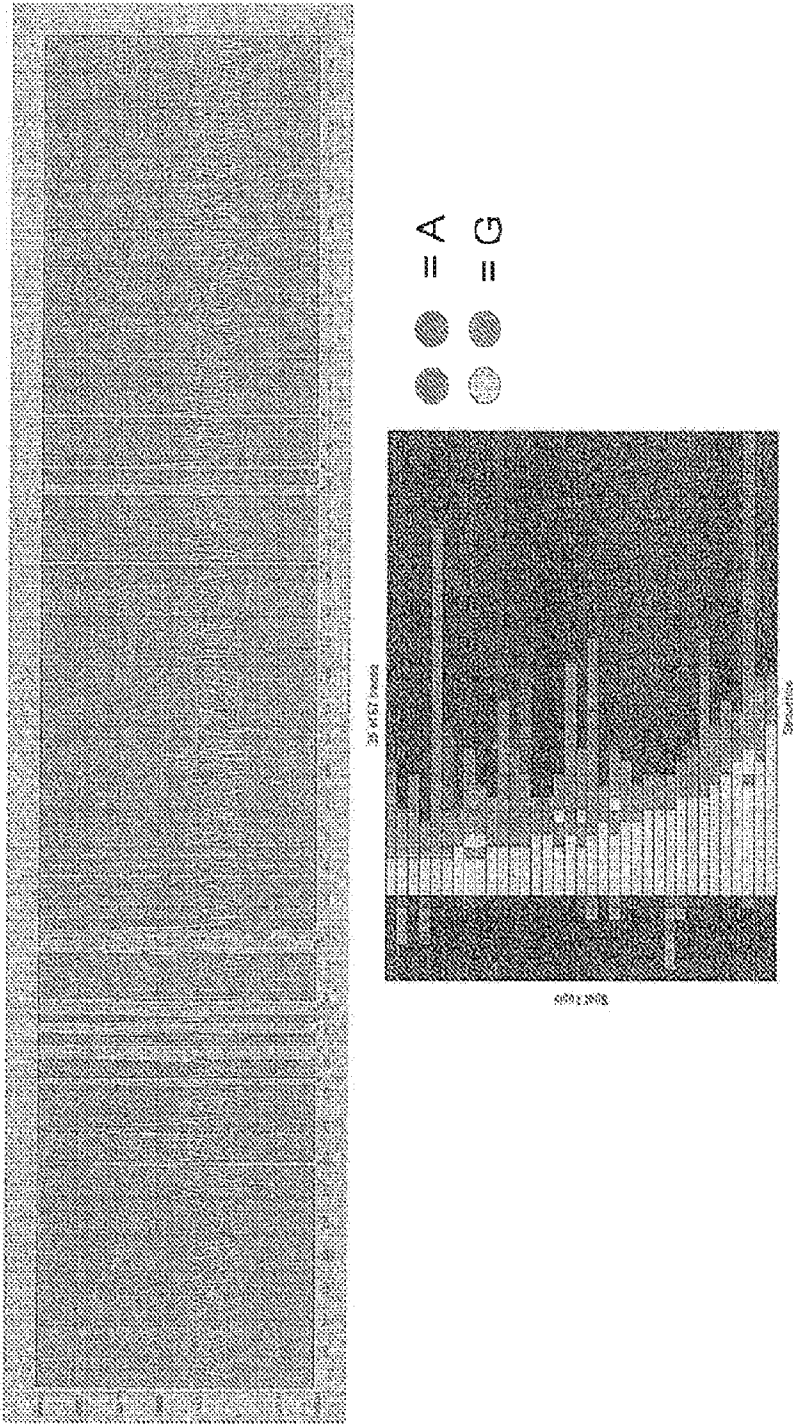


FIG. 14

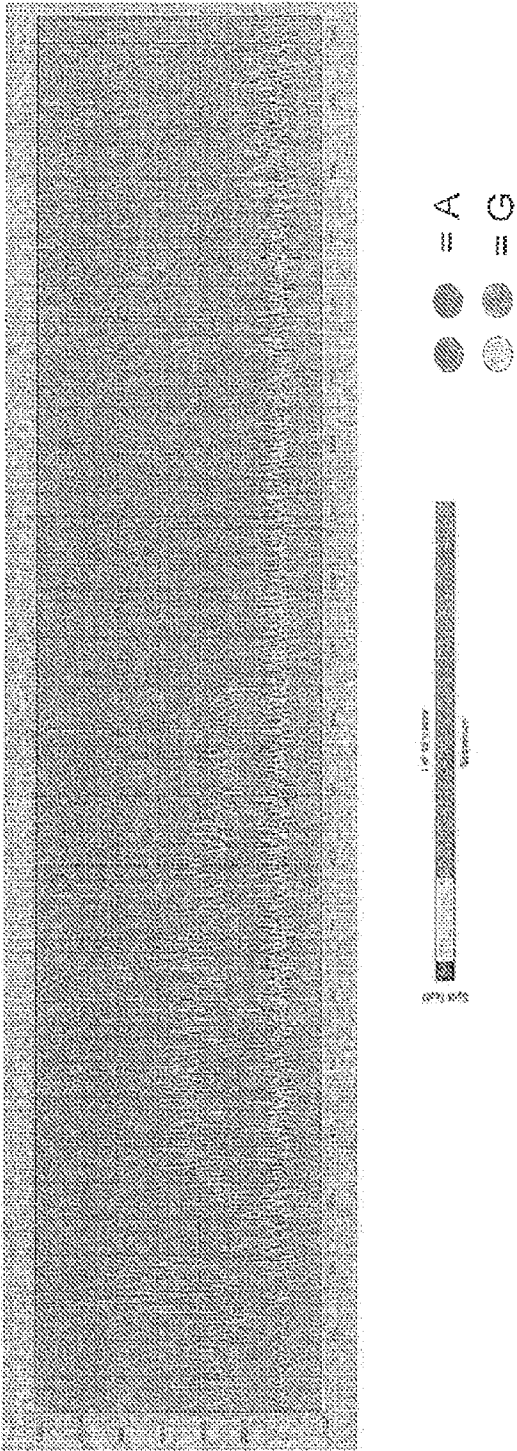


FIG. 15

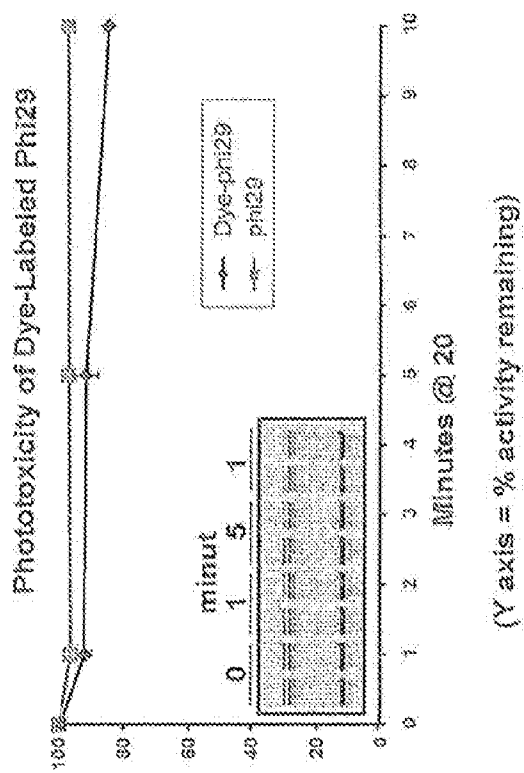
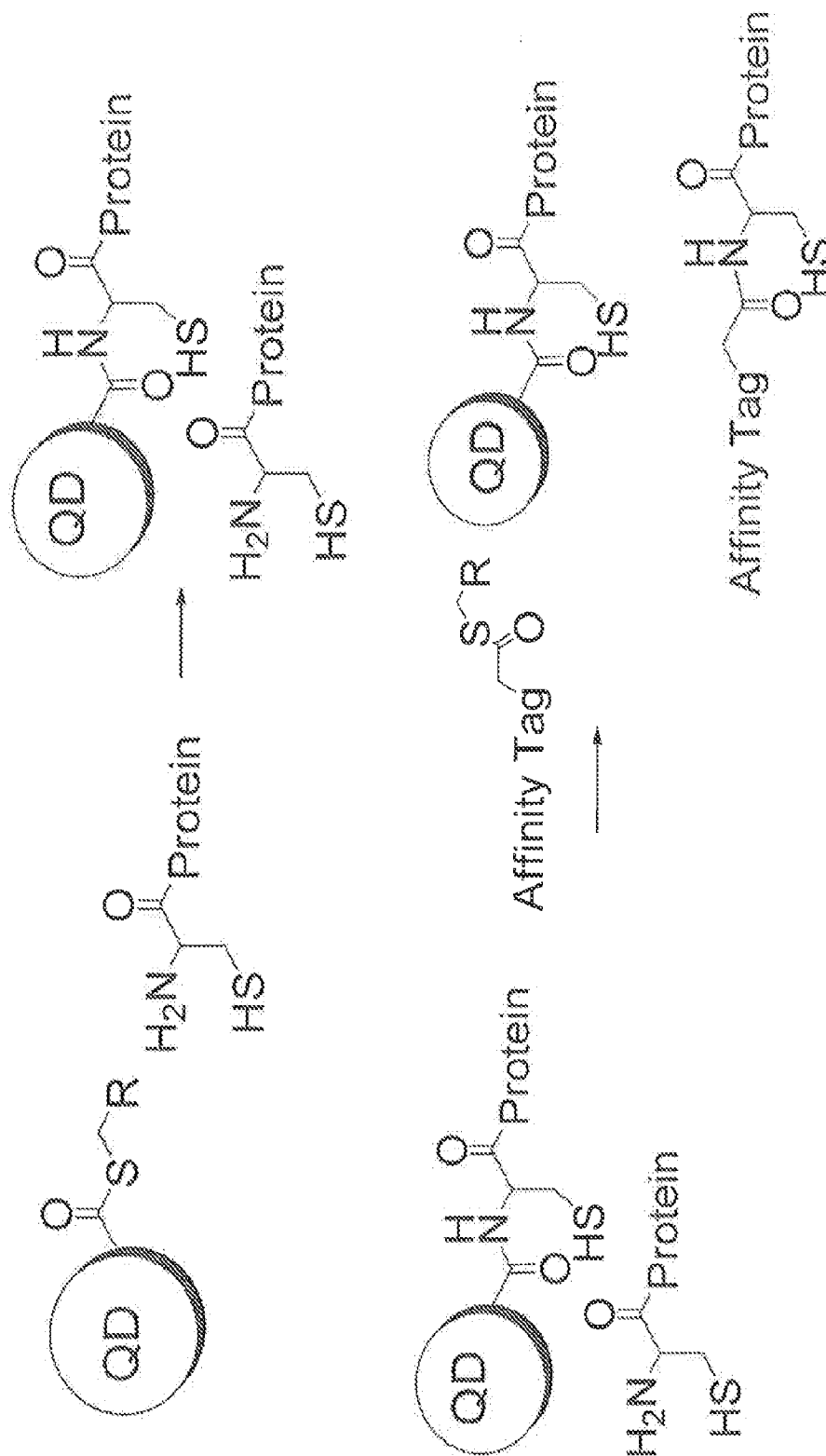
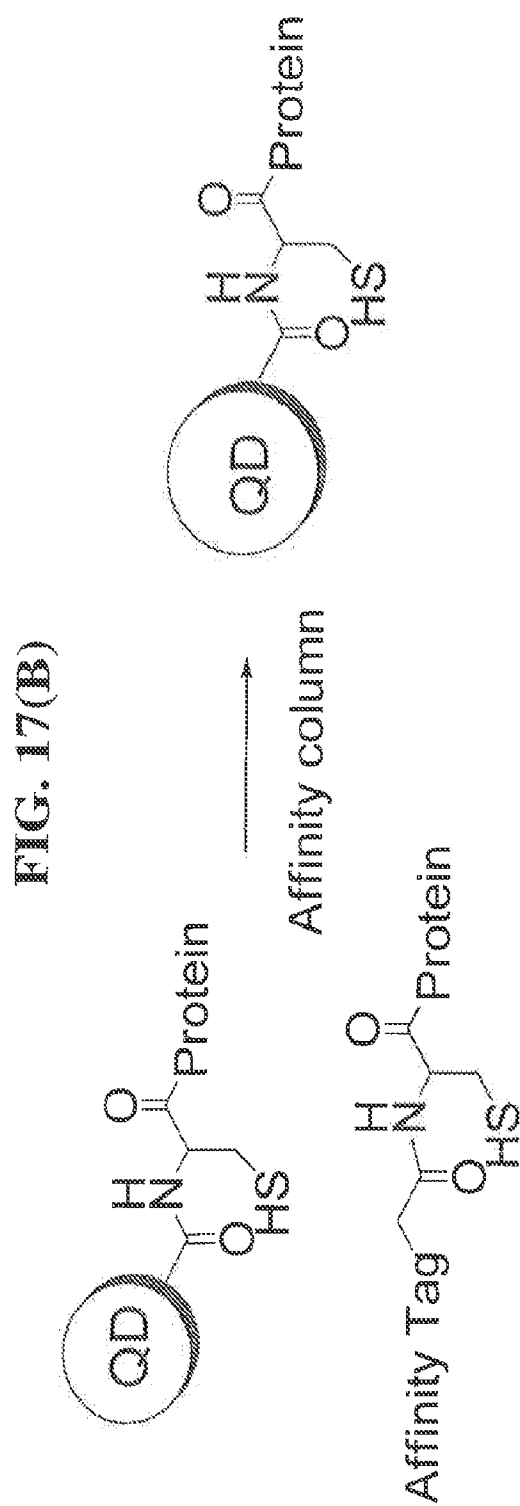


FIG. 16

FIG. 17(A)





Reaction of Aldehydes with N-terminal Cys $\Phi 29$

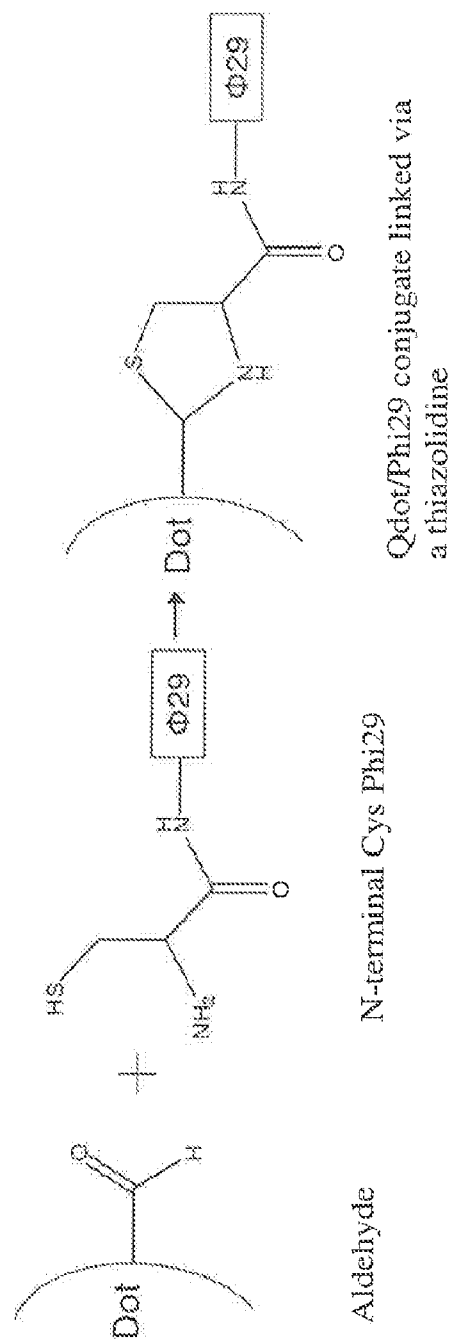
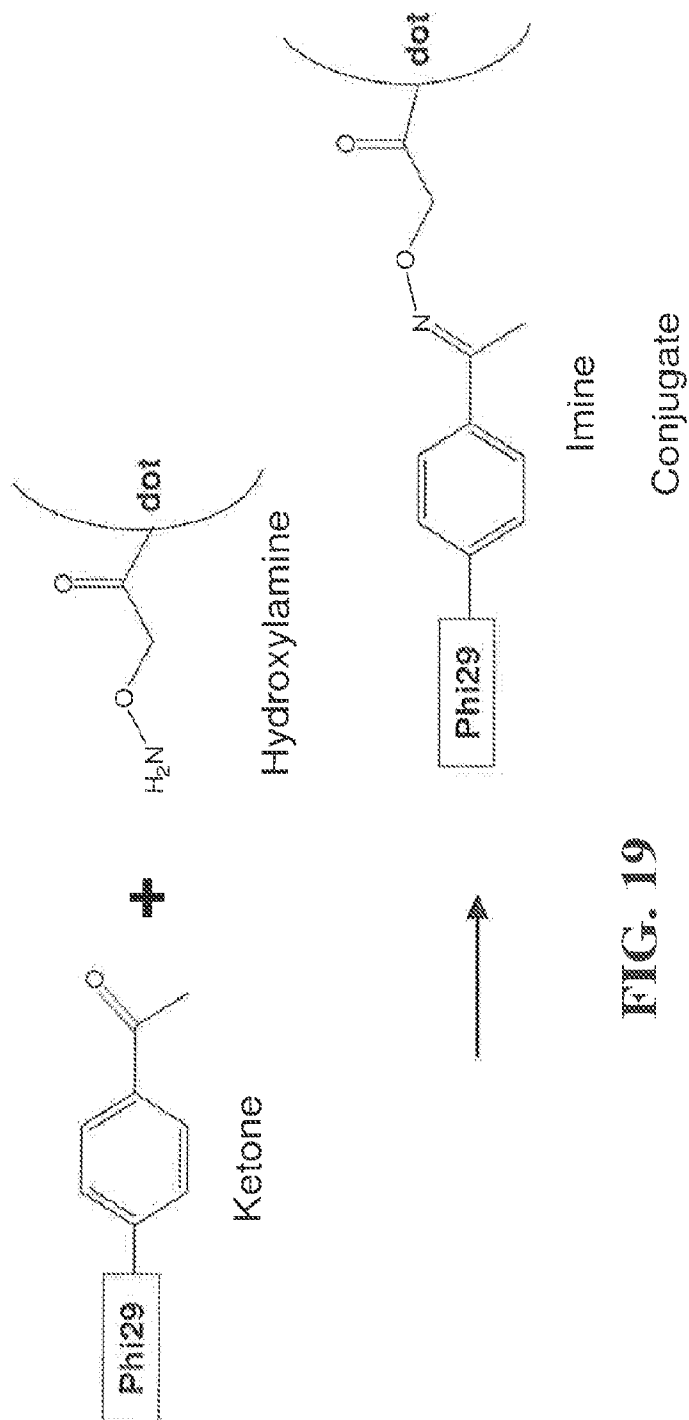


FIG. 18

Conjugation with Nonnatural Amino Acid, Acetylphenyl Alanine (ketone)

**FIG. 19**

**Conjugation with Nonnatural Amino Acid,
Azidophenyl Alanine (azide)**

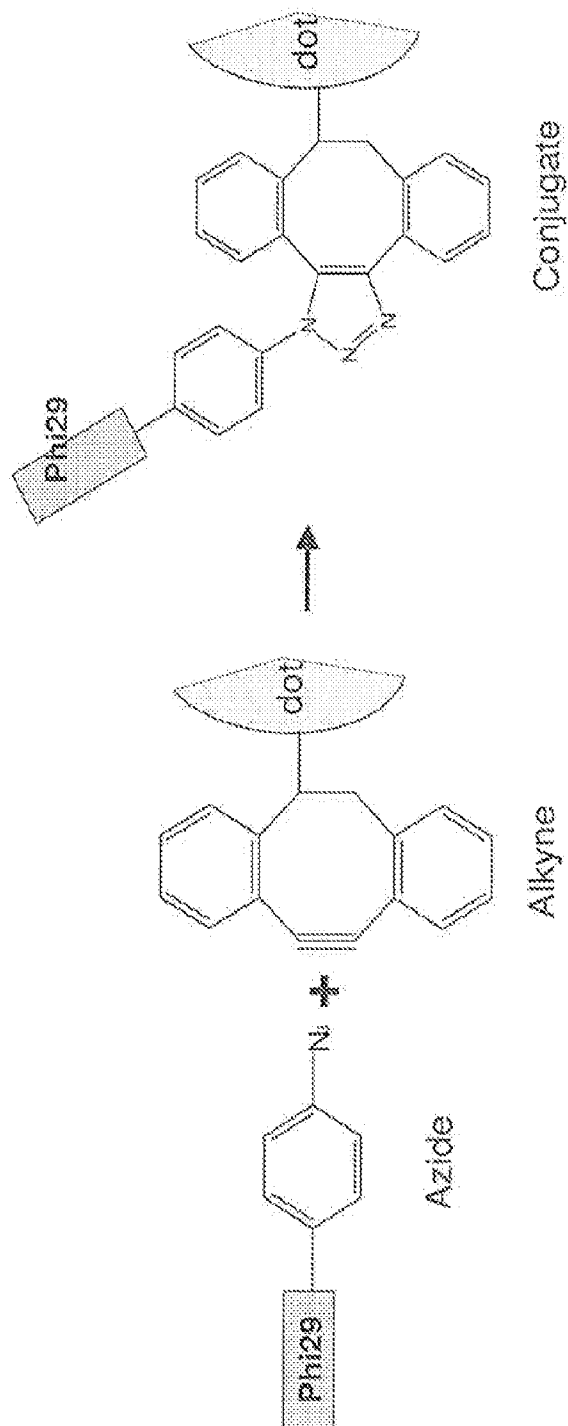


FIG. 20

FIG. 21

