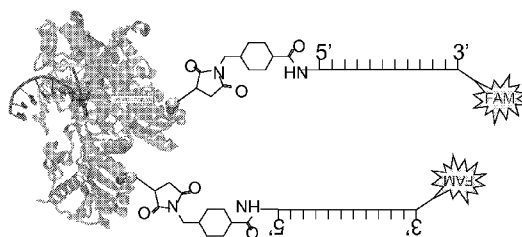




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Bst Large Fragment (1L3U) conjugated to FAM labeled oligo

FIG. 1D

(57) **Abstract:** In some embodiments, the disclosure relates generally to methods as well as related compositions, systems, kits and apparatus comprising linking proteins to target compounds and/or to locations of interest using tethers. For example, the tether can be used to link the protein to a target compound, for example, to link an enzyme to a substrate. Similarly, the tether can be used to link the protein at or near a desired location on a surface. In one group of embodiments, the tether includes a polynucleotide and the target compound or location on the surface includes another polynucleotide that is capable of hybridizing to the tether. In such embodiments, the tether can be used to link the protein to the target compound or location using nucleic acid hybridization.

LINKING METHODS, COMPOSITIONS, SYSTEMS, KITS AND APPARATUSES

BACKGROUND

[0001] The ability of enzymes to catalyze biological reactions is fundamental to life. A range of biological applications use enzymes to synthesize various biomolecules in vitro. One particularly useful class of enzymes are the polymerases, which can catalyze the polymerization of biomolecules (e.g., nucleotides or amino acids) into biopolymers (e.g., nucleic acids or peptides). For example, polymerases that can polymerize nucleotides into nucleic acids, particularly in a template-dependent fashion, are useful in recombinant DNA technology and nucleic acid sequencing applications. Many nucleic acid sequencing methods monitor nucleotide incorporations during in vitro template-dependent replication of a target nucleic acid molecule by a polymerase.

[0002] When using an enzyme to catalyze a biological reaction of interest, it can be useful to confine the enzyme so that it is co-localized with its substrate. Such co-localization can increase the rate or efficiency of enzymatic catalysis, thereby increasing the enzymatic activity and/or product yield under a given set of reaction conditions. Various methods of co-localizing enzymes with substrates, typically by immobilizing the enzyme on a support that is then contacted with a solution including the substrate, have been reported. However, such methods typically cause a reduction in enzyme activity and succeed only at low efficiencies. Such methods typically also require modification of the enzyme prior to or after enzyme immobilization, which can be time-consuming and technically challenging to perform. There remains a need in the art for simple, efficient and reliable methods to tether enzymes to, or in the vicinity of, their target substrates so as to increase the rate or product yield of the enzymatic reaction, as well as more generally to tether proteins to desired locations.

DETAILED DESCRIPTION

[0003] 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 explicitly or implicitly 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.

[0004] The practice of the 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.

[0005] As used herein, the terms “a,” “an,” and “the” and similar referents used herein are to be construed to cover both the singular and the plural unless their usage in context indicates otherwise. Accordingly, the use of the word “a” or “an” when used in the claims or specification, including when used in conjunction with the term “comprising”, may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0006] 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; between a nucleotide and a label; 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).

[0007] In some embodiments, the disclosure relates generally to methods (as well as related compositions, systems, kits and apparatus) comprising link proteins to target compounds and/or to locations of interest using tethers. For example, the tether can be used to link the protein to a target compound, for example, to link an enzyme to a substrate. Similarly, the tether can be used to link the protein at or near a desired location on a surface. In one group of embodiments, the tether includes a polynucleotide and the target compound or location on the surface includes another polynucleotide that is capable of hybridizing to the tether. In such

embodiments, the tether can be used to link the protein to the target compound or location using nucleic acid hybridization.

[0008] Linking proteins to desired targets or locations by exploiting the ability of nucleic acid molecules to selectively hybridize to each other has several advantages. For example, methods of affixing nucleic acids to surfaces, optionally in array format, are well-developed and can easily be adapted for use in protein-based assays. Similarly, the use of nucleic acid hybridization as a tethering mechanism is simple to perform, can be reversed at will via appropriate adjustment of reaction conditions, can be employed using native (i.e., unmodified) nucleic acid molecules and eliminates the need to use binding catalysts or other reactants. Furthermore, by exploiting the ability of polynucleotides to hybridize with each other in a sequence-specific manner, reactions can be performed in multiplex format where different groups of protein are selectively linked to different targets or locations using the same linking conditions.

[0009] The use of tethering mechanisms can also be useful in decreasing the cost or effort associated with performing protein-based applications. For example, many such applications (e.g., enzyme reactions) can consume large amounts of protein, which can be costly and time-consuming to prepare. This problem is aggravated when using proteins in methods that monitor and detect aggregate signals from a population of protein molecules acting upon a population of targets, either in asynchronous (e.g., single molecule) or synchronous format. This problem can be especially intractable when multiple rounds of washing or reagent exchange are involved. In such methods, it can be very costly to repeatedly provide proteins for each fresh round of reaction, particularly at sufficiently high concentrations to permit reaction of the protein with a target. In such situations, the use of tethers to link the protein to the target or to a surface can eliminate loss of proteins during reaction washes, thereby reducing or eliminating the need to replenish the protein in the reaction following each wash. For example, multiple reaction and wash cycles can be performed without consuming large amounts of expensive protein reagents in each wash.

[0010] Tethering can also effectively increase the local concentration of the protein within the zone of reaction with a target, thus effectively increase the rate of reaction and/or increasing the total amount of product formed within a given amount of time. For example, tethering of an enzyme can increase the rate of an enzyme-catalyzed reaction. Typically, such rate is limited by enzyme concentration; tethering limits the ability of the enzyme to diffuse away from the reaction site, effectively increasing the localized protein concentration without requiring the use of very large amounts of protein.

[0011] Finally, use of tethered proteins can sometimes increase the spectrum of reaction conditions available to the user. For example, protein tethering can permit the use of reaction conditions that enhance protein activity but would otherwise cause loss of untethered proteins from the reaction mixture. Described further herein are some exemplary embodiments that further illustrate the various advantages of tethered proteins. For example, use of a tethered polymerase in nucleotide incorporation and/or primer extension applications, such as cyclical (“step-wise”) sequencing-by-synthesis reactions, can reduce the amount of polymerase consumed in each extension step, can increase the reaction rate and/or the total amount of product formed under given reaction conditions and can also permit the use of high-salt reaction conditions without significant loss of polymerase between washes, thus effectively increasing the amount of signal obtained from each extension and reducing the amount of incomplete extensions at each step.

[0012] In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits for linking a protein to a surface, comprising: contacting a tethered protein with a surface, where the tethered protein includes a tether linked to protein, and where the tether of the tethered protein includes a surface-reactive moiety and the surface includes a tether-reactive moiety that is capable of reacting with the surface-reactive moiety; and forming a linkage between the surface-reactive moiety with the tether-reactive moiety, thereby linking the tethered protein to the surface. In some embodiments, the tether reactive moiety and the surface-reactive moiety each comprise one of two complementary members of a binding pair. The binding pair can be selected from a group consisting of: a biotin moiety and an avidin 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 two polynucleotides that are complementary to each other over at least some portion of their respective lengths (where complementarity is optionally defined according to conventional Watson-Crick base pairing rules or alternatively according to some other base-pairing paradigm).

[0013] In some embodiments, the surface-reactive moiety of the tether includes a first polynucleotide having a first polynucleotide sequence, and the tether-reactive moiety of the surface includes a second polynucleotide having a second polynucleotide sequence, where the

first and second polynucleotide sequences are at least 80% complementary to each other (where complementarity is optionally defined according to conventional Watson-Crick base pairing rules or alternatively according to some other base-pairing paradigm). In some embodiments, the first and second polynucleotide sequences are at least 85%, at least 90%, at least 95%, at least 97% or at least 99% complementary to each other.

[0014] In some embodiments, the disclosure relates generally to methods (and related compositions, systems, apparatuses and kits) for linking a protein to a surface, comprising: binding a tethered protein to a surface, where the tethered protein includes a protein linked to a tether, the tether including a first polynucleotide and the first polynucleotide including a first polynucleotide sequence; where the binding further includes contacting the tethered protein with a second polynucleotide, wherein the second polynucleotide is linked to a surface and includes a second polynucleotide sequence that is at least 80% complementary to the first polynucleotide sequence, and hybridizing the first polynucleotide sequence to the second polynucleotide sequence, thereby linking the tethered polymerase to the surface. In some embodiments, the first and second polynucleotide sequences are at least 80% complementary to each other (where complementarity is optionally defined according to conventional Watson-Crick base pairing rules or alternatively according to some other base-pairing paradigm). In some embodiments, the first and second polynucleotide sequences are at least 85%, at least 90%, at least 95%, at least 97% or at least 99% complementary to each other.

[0015] In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits useful for co-localizing an enzyme and its substrate using a tether to link the enzyme to, or in the vicinity of, the substrate. In some embodiments, the colocalization can be performed prior to, or during, the reaction of the enzyme with the substrate. Such co-localization can increase the rate of the enzymatic reaction and/or increase the the product yield.

[0016] In some embodiments, the disclosure relates generally to methods of co-localizing an enzyme with a substrate by linking an enzyme (or any biologically active fragment thereof) to a substrate using a tether, thereby forming an tethered enzyme-substrate complex that includes the enzyme (or biologically active fragment) linked to the substrate through the tether. Typically, the linking is done in such a manner that the enzyme (or biologically active fragment) retains enzymatic activity and can still react with the substrate to form a product after the linking is complete. In one exemplary embodiment, the enzyme-reactive moiety of the tether can be linked to the enzyme (or biologically active fragment) to form a tethered enzyme. The tethered enzyme can be contacted with the substrate under conditions where the

substrate-reactive moiety of the tether in the tethered enzyme binds to the substrate, thereby forming a tethered enzyme-substrate complex that includes the enzyme linked to the substrate through the tether. Optionally, the tethered enzyme-substrate complex retains enzymatic activity. For example, the enzyme (or biologically active fragment) of the tethered enzyme-substrate complex can bind to the substrate within the tethered enzyme-substrate complex, or to any other substrate molecule within the reaction mixture, and can catalyze the enzyme-substrate reaction. In some embodiments, the substrate is linked to a surface, such that formation of the tethered enzyme-substrate complex effectively localizes the enzyme (or biologically active fragment) to the surface.

[0017] In some embodiments, the tether is not used to link the enzyme to the substrate, but is instead used to link the enzyme to a surface. For example, the tether can link the enzyme to the surface, thereby forming a surface-linked tethered enzyme, while the substrate can be free-floating or independently attached to the same or different surface. In some embodiments, the substrate is linked to the same surface as the the tethered enzyme. For example, the enzyme and substrate can each be independently linked to the same surface at attachment sites that are sufficiently close to each other, and where the tether is sufficiently flexible, to permit reaction of the enzyme with the substrate.

[0018] In some embodiments, the disclosure relates generally to methods (and related compositions, systems, kits and apparatuses) for co-localizing an enzyme and its substrate comprising: forming a tethered enzyme by linking a tether to the enzyme (or biologically active fragment thereof), where the tethered enzyme retains enzymatic activity; and binding the tethered enzyme to a substrate. Optionally, the tether includes an enzyme-reactive moiety, and the linking further includes reacting the enzyme-reactive moiety with the enzyme, thereby forming an enzyme-tether linkage that links the tether to the enzyme. The linkage can be a covalent linkage, an electrostatic linkage or any other linkage suitable for linking the enzyme (or biologically active fragment) to the tether. In some embodiments, the tether includes a substrate-reactive moiety, and the disclosed methods (and related compositions, systems, kits and apparatuses) further involve linking the tether to the substrate by reacting the substrate-reactive moiety with the substrate, and the linking further includes reacting the substrate-reactive moiety with the substrate, thereby forming an substrate-tether linkage that links the tether to the substrate. In some embodiments, the enzyme in the tethered enzyme selected from the group consisting of: a polymerase, a ribosome, a helicase, a pyrophosphatase and an apyrase.

[0019] In some embodiments, the disclosure relates generally to methods (and related compositions, systems, apparatuses and kits) for linking polymerases to target compounds, or to desired locations of interest. In some embodiments, the polymerase can include that is capable of catalyzing the incorporation of one or more nucleotides into a nucleic acid molecule. Typically but not necessarily such nucleotide incorporation can occur in a template-dependent fashion. The polymerase can be a naturally-occurring polymerase, or any subunit or truncation thereof, a mutant, variant, derivative, recombinant, fusion, modified, chemically modified, or otherwise engineered form of any polymerase, a synthetic molecule or assembly that can catalyze nucleotide incorporation, as well as analogs, derivatives or fragments thereof that retain the ability to catalyze such nucleotide incorporation. 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, 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 including both DNA-dependent and RNA-dependent DNA polymerases (such as for example Bst DNA polymerase, Terminator™ polymerase, KOD polymerase, Phi-29 DNA polymerase, reverse transcriptases and *E. coli* DNA polymerase) and RNA polymerases. In some embodiments, the polymerase is a 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. In one exemplary embodiment, the 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.

[0020] Typically, the polymerase includes a nucleic acid binding site and a polymerase active site. The polymerase active site can be a site of polymerase activity. The polymerase activity can comprise any *in vivo* or *in vitro* enzymatic activity of the polymerase that relates to catalyzing the incorporation of one or more nucleotides into a nucleic acid molecule, for example, 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 possess other activities such as DNA binding activity, 3' to 5' or 5' to 3' exonuclease activity, and the like.

[0021] In some embodiments, the disclosure relates generally to methods (and related compositions, systems, kits and apparatuses) for linking a polymerase to a surface, comprising: contacting a tethered polymerase with a surface, where the tethered polymerase includes polymerase linked to a tether including a first polynucleotide, where the polynucleotide has a first polynucleotide sequence, and the surface includes a second polynucleotide including a second polynucleotide sequence, where the first and second polynucleotide sequences are at least 80% complementary to each other; and hybridizing the first polynucleotide sequence to the second polynucleotide sequence, thereby linking the tethered polymerase to the surface.

[0022] In some embodiments, the first polynucleotide of the tether is covalently linked to an amino acid residue of the polymerase. For example, the first polynucleotide can be covalently linked to a cysteine residue of the polymerase. The first polynucleotide can be covalently linked to the cysteine residue through a flexible linker.

[0023] Typically, the tethered polymerase has polymerase activity, and can catalyze the incorporation of one or more nucleotides into a nucleic acid molecule.

[0024] In some embodiments, the disclosed methods (and related compositions, systems, kits and apparatuses) can further include contacting the tethered polymerase with one or more nucleotides.

[0025] In some embodiments, the disclosed methods (and related compositions, systems, kits and apparatuses) can further include incorporating one or more nucleotides into the second polynucleotide using the tethered polymerase. For example, the tethered polymerase can polymerize the one or more nucleotides onto the 3' end of the second polynucleotide.

[0026] In some embodiments, the surface includes a third polynucleotide, and the disclosed methods (and related compositions, systems, kits and apparatuses) can further include incorporating one or more nucleotides into the third polynucleotide using the tethered polymerase. For example, the tethered polymerase can polymerize the one or more nucleotides onto the 3' end of the third polynucleotide.

[0027] In some embodiments, the disclosure relates generally to compositions (as well as related methods, systems, kits and apparatus) comprising tethered proteins, where the tether can be fixed to a site of interest. For example, the tether can link the protein to a substrate or to a surface.

[0028] In some embodiments, the disclosure relates generally to compositions (and related methods, systems, kits and apparatuses) comprising tethered enzymes. Typically, the tethered enzyme includes an enzyme linked to a tether, where the tethered enzyme has enzymatic activity.

[0029] In some embodiments, the tether is a linear tether. In some embodiments, the tether can include a linear polymer, which can be rigid or flexible. In some embodiments, the tether includes a polynucleotide. In some embodiments, the tether includes a flexible linker. In some embodiments, the flexible linker can have a persistence length of up to about 200 nm; for example between about 0.5 nm and about 1500 nm; typically between about 1.0 nm and about 15 nm or between about 1.5 nm and about 10 nm; even more typically between about 1.5 nm and 5.0 nm, where the persistence length is measured under any reaction conditions that are suitable for primer extension (for example, in a buffer comprising between 0 and about 2M salt (e.g., NaCl or MgCl₂)), even more typically in a buffer comprising between 0 and about 100 mM salt (e.g., NaCl or MgCl₂). Methods of measuring persistence length of polynucleotides in solution are known in the art; see, e.g., Murphy et al., "Probing Single Stranded DNA Conformational Flexibility Using Fluorescence Spectroscopy", *Biophys J.* 2004 April; 86(4): 2530--2537. In some embodiments, the flexible linker includes a polynucleotide. In some embodiments, the flexible linker includes a polypeptide. In some embodiments, the flexible linker includes polyethylene glycol.

[0030] Optionally, the tether can include one or more labels. The one or more labels can be detected using any suitable detection system. For example the one or more labels can include fluorescent labels, luminescent labels, chemically detectable labels, or magnetically detectable labels.

[0031] In some embodiments, the tether can include one or more reactive moieties. In some embodiments, the tether includes an enzyme-reactive moiety. The enzyme-reactive moiety can react with the enzyme under suitable conditions. Reaction of the enzyme-reactive moiety with the enzyme can result in the formation of an enzyme-tether linkage that links the tether to the enzyme, thereby forming a tethered enzyme. The enzyme-tether linkage can include one or more bonds selected from the group consisting of: a covalent bond, an electrostatic bond, an affinity-based interaction and a hydrogen bond. In some embodiments, the enzyme-reactive moiety of the tether reacts with a sulfhydryl group of a cysteine residue of the enzyme and forms a covalent bond with a sulfur atom of the sulfhydryl group of the cysteine. In some embodiments, the enzyme-reactive moiety of the tether reacts with an amino group of an amino acid residue of the enzyme. The amino group can be located on an N-terminal amino acid residue of the enzyme. In some embodiments, the enzyme-reactive moiety of the tether reacts with a carboxyl group of an amino acid residue of the enzyme. The carboxyl group can be located on a C-terminal amino acid residue of the enzyme.

[0032] In some embodiments, the enzyme includes a reactive sulfhydryl group (for example, a sulfhydryl group of a cysteine residue, that is optionally a surface cysteine) and the enzyme-reactive moiety of the tether includes a linking group that is capable of reacting with the sulfhydryl group of the cysteine residue. For example, the linking group can include a reactive amine that can be activated by suitable treatment (e.g., reaction with SMCC) to form a reactive maleimide. The tether including the reactive maleimide can then be contacted with the enzyme under suitable conditions where the reactive maleimide group reacts with the sulfhydryl group of the cysteine, forming a covalent linkage, typically a linkage comprising a thioether bond.

[0033] In some embodiments, the tether includes a substrate-reactive moiety. The substrate-reactive moiety can react with the substrate under suitable conditions. For example, the substrate-reactive moiety can react or bind selectively to the substrate when contacted with the substrate. Reaction of the substrate-reactive moiety with the substrate can result in the formation of a substrate-tether linkage that links the tether to the substrate. The substrate-tether linkage can include one or more bonds selected from the group consisting of: a covalent bond, an electrostatic bond, an affinity-based interaction and a hydrogen bond. In one exemplary embodiment, the substrate-reactive moiety of the tether comprises a first polynucleotide including a first polynucleotide sequence, and the substrate includes, e.g., is linked to, a second polynucleotide including a second polynucleotide sequence, where the first and second polynucleotide sequences are at least 80% complementary to each other. When the tether and substrate are contacted with each other under hybridization conditions, then first and second polynucleotide sequences hybridize to each other, thereby forming a nucleic acid duplex that links the tether to the substrate.

[0034] In some embodiments, the tether of the tethered enzyme includes a surface-reactive moiety. The surface-reactive moiety can react with a surface under suitable conditions. Reaction of the surface-reactive moiety with the surface can result in the formation of a surface-tether linkage that links the tether to the surface. The surface-tether linkage can include one or more bonds selected from the group consisting of: a covalent bond, an electrostatic bond, an affinity-based interaction and a hydrogen bond.

[0035] In some embodiments, the surface-reactive moiety of the tethered enzyme includes a first polynucleotide, and the surface includes a second polynucleotide. The first and second polynucleotides may be capable of hybridizing to each other over at least a portion of their respective lengths. In some embodiments, the first polynucleotide includes a first polynucleotide sequence and the second polynucleotide includes a second polynucleotide sequence, where the first and second polynucleotide sequences are at least 80%

complementary to each other. In some embodiments, the first and second polynucleotide sequences are at least 80% complementary, at least 90% complementary, at least 95% complementary, at least 97% complementary, or at least 99% complementary to each other. Typically, complementarity can be defined according to conventional Watson-Crick base pairing rules (e.g., A base pairs with T and C base pairs with G); alternatively complementarity can be defined according to non-Watson Crick base pairing paradigms as well. In some embodiments, the first polynucleotide sequence and the second polynucleotide sequence are hybridized to each other.

[0036] In some embodiments, the tether is linked to any one or more members of the group consisting of: enzyme, substrate and surface. For example, the tether can first be linked to the enzyme through the enzyme-reactive moiety of the tether, thereby forming a tethered enzyme, and the tether of the tethered enzyme can then be linked to a substrate using the substrate-reactive moiety of the tether, or alternatively linked directly to a surface using a surface-reactive moiety of the tether. Alternatively, the tether can first be linked to a substrate, or to a surface, and then be linked to the enzyme.

[0037] In a typical embodiment, the enzyme comprises a polymerase.

[0038] In some embodiments, the disclosure relates generally to a composition comprising a tethered polymerase including a polymerase linked to a tether, where the tethered polymerase has polymerase activity. The polymerase-tether linkage can include one or more bonds selected from the group consisting of: a covalent bond, an electrostatic bond, an affinity-based interaction and a hydrogen bond. In some embodiments, the polymerase can be covalently linked to the tether. The tether can be linked to an amino acid residue of the polymerase. In some embodiments, the tether is linked to a sulfhydryl group of a cysteine residue of the polymerase. In some embodiments, the tether is linked to an amino group of an amino acid residue of the polymerase. In some embodiments, the tether is linked to a carboxyl group of an amino acid residue of the polymerase. The tether can include a polynucleotide. The polymerase can be covalently linked to the polynucleotide. The polynucleotide can be covalently linked to a cysteine residue of the polymerase. In some embodiments, a 5' end of the polynucleotide is covalently linked to an amino acid residue of the polymerase. The covalent linkage between the tether and the polymerase can include a series of atoms, each atom in the series being linked covalently to the next atom in the series such that the tether and the polymerase are ultimately linked to each other through a series of atoms linked through covalent bonds.

[0039] In some embodiments, the polymerase of the tethered polymerase is linked to a first member of a binding pair, the tether is linked to a second member of the binding pair, and where the first member and the second member are linked to each other to form the tethered polymerase. The binding pair can be selected from a group consisting of: a biotin moiety and an avidin 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 two polynucleotides that are complementary to each other over at least some portion of their respective lengths (where complementarity can optionally be defined according to conventional Watson-Crick base pairing rules or alternatively according to some other base-pairing paradigm).

[0040] 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 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, CNH₂, C(O), C(CH₂), CH₂CH₂, or C(OH)CH₂R (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, BH₃, or S. In the phosphorus chain, a phosphorus atom with a side group 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 (e.g., reporter moiety) 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 or substitute 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, 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.

[0041] As used herein, the term “nucleotide incorporation” and its variants comprise polymerization of one or more nucleotides to form a nucleic acid strand including at least two nucleotides linked to each other, typically but not necessarily via phosphodiester bonds, although alternative linkages may be possible in the context of particular nucleotide analogs.

[0042] The following non-limiting examples are provided purely by way of illustration of exemplary embodiments, and in no way limit the scope and spirit of the present disclosure. Furthermore, it is to be understood that any inventions disclosed or claimed herein encompass all variations, combinations, and permutations of any one or more features described herein. Any one or more features may be explicitly excluded from the claims even if the specific exclusion is not set forth explicitly herein. It should also be understood that disclosure of a reagent for use in a method is intended to be synonymous with (and provide support for) that method involving the use of that reagent, according either to the specific methods disclosed herein, or other methods known in the art unless one of ordinary skill in the art would understand otherwise. In addition, where the specification and/or claims disclose a method, any one or more of the reagents disclosed herein may be used in the method, unless one of ordinary skill in the art would understand otherwise.

EXAMPLES

[0043] Example 1: Construction of a tethered polymerase

[0044] A tethered polymerase was constructed by covalently linking a tether including a first polynucleotide sequence to a cysteine residue of a variant Bst polymerase (“Ion Sequencing Polymerase”) having the following amino acid sequence:

```
MAKMAFTLADRVTEEMLADKAALVVEVVEENYHDAPIVGIAVVNERGRFFLRPETALADPQFVAWLGDETKKKSMF
DSKRAAVALKWKGIELCGVSFDLLLAAYLLDPAQGVDDVAAAAMKQYEAVRPDEAVYGKGAKRAVPDEPVLAEHL
VRKAAAIWELERPFDELRRNEQDRLLVELEQPLSSILAEMEFAGVKVDTKRLEQMGKELAEQLGTVEQRIYELAG
QEFNINSPKQLGVILFEKLQLPVLKKTGTGYSTSADVLEKLAPYHEIVENILHYRQLGKLQSTYIEGLLKVVRPDT
KKVHTIFNQALTQTGRLSSTEPNLQNIPIRLEEGRKIRQAFVPSESDWLIFAADYSQIELRVLAHIAEDDNLMEAF
RRDLDIHTKTAMDIFQVSEDEVTPNMRRQAKAVNFGIVYGISDYGLAQNLSRKEAAEFIERYFQSFPGVKRYME
NIVQEAKQKGYVTLLHRRRYLPDITSRNFNVRSAERMAMNTPIQGSAADIKKAMIDLNARLKEERLQAHLQLQ
VHDELILEAPKEEMERLCRLVPEVMEQAVTLRVPLKVDYRYGSTWYDAK
```

[0045] The tether included a polynucleotide sequence complementary to the sequence of a sequencing primer P1 (described further below) and a flexible linker including a poly-A stretch, as well as a primary amine at the 5' end of tether. The 3' end of the tether was blocked using a FAM group. The structure of the tether is depicted in FIG. 1A.

[0046] The primary amine of the tether can be activated via conversion to a maleimide group using SMCC, as shown in FIG. 1B. The reaction and resulting product is depicted in FIG. 1C.

[0047] The tether of FIG. 1 was then covalently linked to two cysteine residues of the Bst Polymerase to obtain the tethered polymerase depicted in FIG. 1D according to the methods described below.

[0048] Activation of the tether

[0049] The following reagents were used:

[0050] The tether including a 49 base-pair oligonucleotide with a tB30 amino tether and FAM; MW = 16115.9; 105.7 nm = 1.7 mg (IDT DNA). The structure and primary amino acid sequence of the tether can be depicted as follows:

[0051] 5AmMC12/AA AAA AAA AAA AAA AAA AAA GAC TGC CAA GGC ACA
CAG GGG ATA GGA AA/36-FAM

[0052] Bst; Ion Torrent lot 7 sequencing polymerase, ~ 1 mg/mL

[0053] Klenow (KF) exo- wt (106.9 µM; 7.6 mg/mL; Starlight source)

[0054] AlexaFluor 647 maleimide (1 mg; A20347; MW~1300)

[0055] AlexaFluor 647 cadaverine (1 mg; A30679; MW ~ 1000)

[0056] SMCC (Molecular Probes; S1534; Lot: 38985A; MW = 334.33)

[0057] sulfo-SMCC (Molecular Biosciences; MW = 463.37)

[0058] sodium bicarbonate (J.T. BAKER; 3506-1; FW 84.01)

[0059] The Bst polymerase was then labeled with maleimide dye (AF647 maleimide).

Results are depicted in FIG. 2.

[0060] The tether including the FAM-labeled oligonucleotide was activated with SMCC to generate a maleimide-oligo-FAM product. Representative results of the activation reaction are depicted in FIG. 3.

[0061] The maleimide-oligo-FAM product was purified with NAP-5 column

[0062] Two different polymerases, Klenow fragment ("KF") and the variant Bst polymerase ("Bst") were reacted with maleimide-oligo-FAM to form tethered polymerases, and the reaction products were purified on a gel. Representative results are depicted in FIG. 4. A possible product band was detected using gel analysis using less than 10% of the reaction.

[0063] Linkage of the polymerase to the tether

[0064] The following protocol was used to link the polymerase to the tether:

[0065] **(a) Polymerase DTT treatment and purification to reduce disulfide bonds:**

[0066] **Reagents (stock concentration) and Materials:** Lot 7 sequencing Bst polymerase (~1 mg/mL), DTT (1 M),

[0067] Tris pH 8.5 (1 M), Exchange Buffer (50 mM Mes pH 6.0, 2 mM EDTA) NAP-5 column (G.E. NAP column, product# 17-0853-02),

[0068] Amicon 30K centrifugal filter (Millipore Cat No. UFC503096 Lot No. ROMA72710), 1-1.5 mL Eppendorf Tubes

[0069] **Protocol:** Mix Bst polymerase (25 μ L), DTT (1 μ L), Tris pH 8.5 (5 μ L) and ddH₂O (69 μ L). Incubate at 4°C for 1-12 h. Purify Bst polymerase using a NAP-5 column. Use gravity flow to prepare and use the NAP-5 column: (1) drain storage buffer (2) add exchange buffer and pass >15 mL exchange buffer (3) add reaction mix and drain until all of the mix has entered the matrix and (4) fill column with exchange buffer and (6) collect 5 drop fractions. Next use the Nanodrop to determine in which fraction(s) contain the Bst polymerase. Pool the fractions and concentrate using the Amicon 30K centrifugal filter using the manufacture's instructions.

[0070] (Amicon Protocol: spin for 7 min at 11,000xg. Transfer filter to clean collection tube and insert in an inverted manner. Spin for 1 min at 1000xg). Measure the absorbance of the concentrated Bst polymerase using the Nanodrop and determine the Bst concentration using the following formula:

[0071] $\epsilon_{280} = 58000/\text{Mcm}$.

[0072] The reaction is depicted in FIG. 5.

[0073] **(b) Activation of FAM-oligo with SMCC**

[0074] The oligo tether was activated using SMCC to generate a FAM-oligo derivatized with maleimide

[0075] **Reagents:**

[0076] SMCC (MW = 334.33g/mol; Molecular Probes; product #:S1534; Lot:38985A),

[0077] Sodium bicarbonate (J.T. Baker; product #:3506-01)

[0078] FAM-oligo (tB30 Amino tether FAM; MW = 16115.9; 105.7 nm = 1.7 mg),

[0079] DMSO

[0080] **Protocol:** FAM-oligo was dissolved in ddH₂O to obtain a 500 μ M concentration.

Approximately 1 mg of SMCC was weighed and dissolved with DMSO to prepare a 20 mM concentration. The following reaction mix was prepared: FAM-oligo (20 μ L), NaHCO₃ (20 μ L) and SMCC (10 μ L). The reaction was incubated at room temperature for 20-30 min. The product was purified using a NAP-5 column. The same protocol to purify the polymerase was used to purify the product. The fractions were identified with UV-Vis (nanodrop). An aliquot of the identified fraction was diluted with an equal amount of 250 mM phosphate (pH 9) to measure the absorbance of FAM and use a molar extinction coefficient of $\epsilon_{510} = 58000/\text{Mcm}$ to determine the FAM concentration. Analytical HPLC confirmed that the desired product. However, an additional peak was also observed. This corresponding peak is probably the product with a hydrolyzed maleimide. Reducing the reaction pH and time minimizes the production of this product.

[0081] The reaction is depicted in FIG. 6.

[0082] **(c) Linking the tether to the polymerase**

[0083] The tether was linked to the polymerase to form a tethered polymerase as follows:

[0084] **Reagents:**

[0085] Bst (DTT treated and purified),

[0086] FAM-oligo-Maleimide (freshly prepared)

[0087] Exchange buffer (50 mM MES pH 6.0, 2 mM EDTA or 50 mM ACES pH 6.8, 2 mM EDTA)

[0088] NaCl

[0089] **Protocol:**

[0090] Mix Bst and FAM-oligo-maleimide in exchange buffer in the presence of 0.5-1 M NaCl.

[0091] Incubate reaction at 4°C for ~ 12h

[0092] The reaction is depicted in FIG. 7.

[0093] As depicted in FIG. 8, the use of varying salt concentrations in the exchange buffer resulted in different product yields, with the inclusion of NaCl pushing the reaction yield to greater than 50%.

[0094] EXAMPLE 2: USE OF TETHERED POLYMERASE FOR NUCLEIC ACID SEQUENCING

[0095] The tethered polymerase of the prior Example, comprising a Bst polymerase linked to an oligonucleotide tether, was used in an ion-based sequencing reaction using the Ion Torrent PGM sequencing platform (Life Technologies).

[0096] Both tethered polymerase and corresponding untethered polymerase (control) were bound to ssDNA beads and washed with high salt. The sequencing primer was then hybridized to the ssDNA template. Following hybridization, the beads were run in a standard sequencing reaction on the Ion Torrent PGM Sequencing platform. As depicted in FIG. 9 and FIG. 10 sequencing using high-salt washes between successive extensions in the PGM platform was observed to be supported by the tethered polymerase of Example 1, but not with the corresponding (control) untethered polymerase. The use of high salt buffers is advantageous because it increases polymerase activity.

[0097] As depicted in FIG. 9, the Ion Sequencing Polymerase tethered with an oligo as described in Example 1, where the tether immobilizes the tethered polymerase to the ssDNA template, was observed to remain on the Ion beads under high salt conditions as the tethered polymerase was not washed off in these conditions. High salt is not expected not to harm the polymerase except to wash it from the ssDNA.

[0098] As depicted in FIG. 10, the same Ion Sequencing Polymerase (in untethered form) was observed to bind Ion Spheres (beads) with ssDNA template in the Ion W2 reagent (6.3mM MgCl₂, 13mM NaCl, 0.01% Triton X-100, pH 7.5) with low affinity and could be washed off with increasing ionic strength. At 1M NaCl, 100% of Ion Sequencing Polymerase 1.0 was washed off Ion Spheres.

[0099] The performance of the Ion Sequencing Polymerase in both tethered and untethered (control) forms in an Ion PGM Sequencing system was measured and compared.

[00100] The untethered form of the Ion Sequencing Polymerase exhibited no signal using high salt sequencing conditions (20 mM MgCl₂, 200 mM NaCl, 0.01% Triton X-100, pH 7.5), and no observable sequencing reads were obtained.

[00101] In contrast, the tethered Ion Sequencing Polymerase demonstrated robust sequencing performance using the high salt sequencing conditions (20 mM MgCl₂, 200 mM NaCl, 0.01%

Triton X-100, pH 7.5), where over 800,000 Q17 reads were obtained and over 30 key signals detected from a single sequencing reaction. As these results indicate, PGM sequencing at high salt (>200mM ionic strength) is supported by the tethered Ion Sequencing Polymerase but not by the untethered Ion Sequencing Polymerase.

What is claimed:

1. A composition comprising a protein tethered to a substrate.
2. The composition of claim 1, wherein the tether comprises a first polynucleotide having a first polynucleotide sequence hybridized to a second polynucleotide having a second polynucleotide sequence.
3. The composition of claim 1, wherein the first polynucleotide is attached to the protein.
4. The composition of claim 1, wherein the second polynucleotide is attached to the substrate.
5. The composition of claim 1, wherein the protein comprises an enzyme.
6. The composition of claim 1, wherein the protein comprises a polymerase enzyme.
7. The composition of claim 1, wherein the protein comprises a DNA polymerase enzyme.
8. A composition comprising a protein linked to a tether, the tether including a first polynucleotide and the first polynucleotide including a first polynucleotide sequence, wherein the linking further includes contacting the tethered protein with a second polynucleotide, wherein the second polynucleotide is linked to a surface

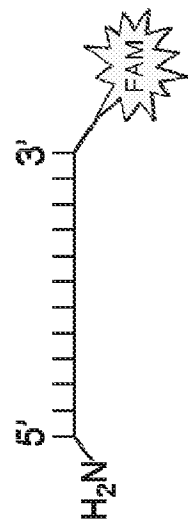
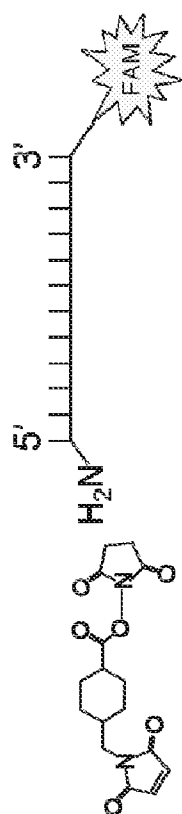


FIG. 1A

**FIG. 1B**

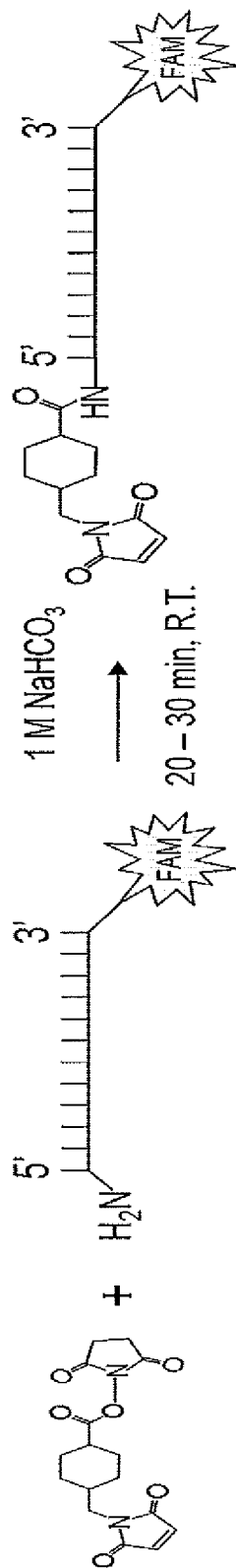
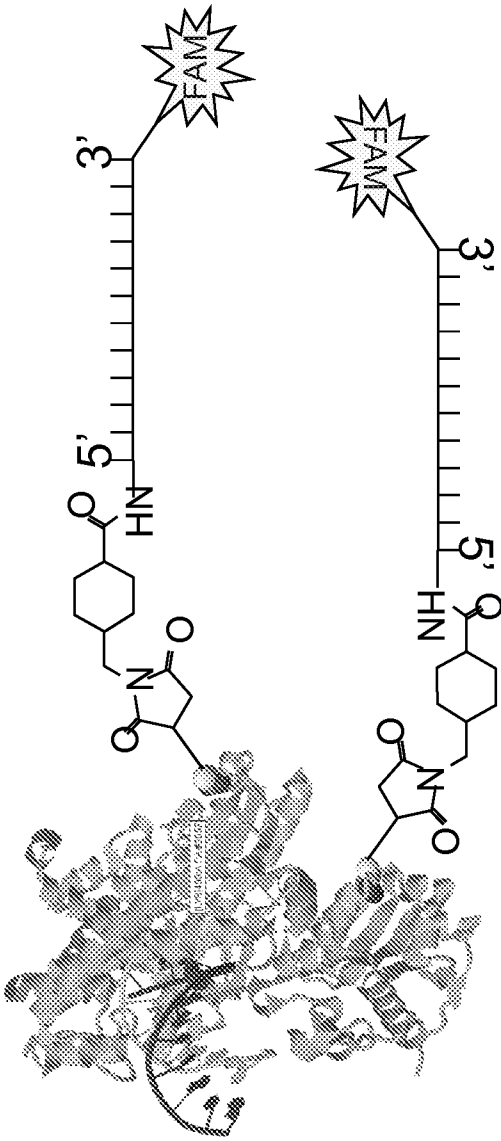


FIG. 1C



Bst Large Fragment (1L3U) conjugated to FAM labeled oligo

FIG. 1D

Polymerase labeling with AF647 maleimide dye

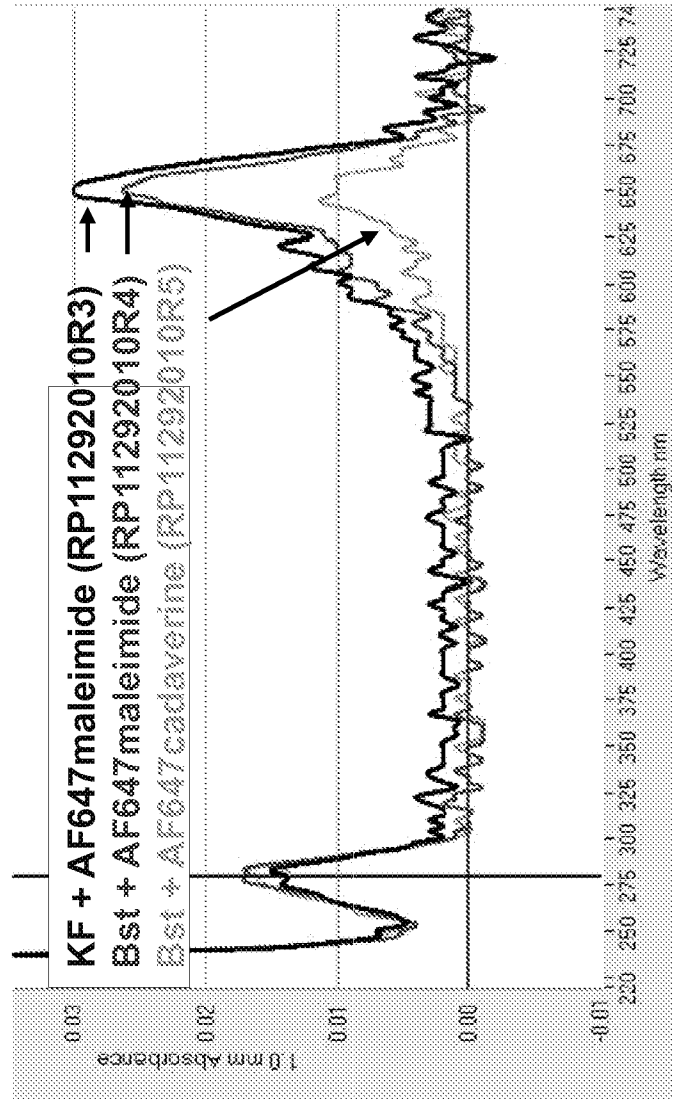


FIG. 2

reaction	A280	A647	[protein](M)	ratio moles of dye/mole protein
KF + AF647maleimide	0.14	0.3	2.07937E-06	0.60
Bst + AF647maleimide	0.17	0.25	2.80172E-06	0.37
Bst + AF647cadaverine	0.16	0.1	2.7069E-06	0.15

FAM-oligo SMCC activation results

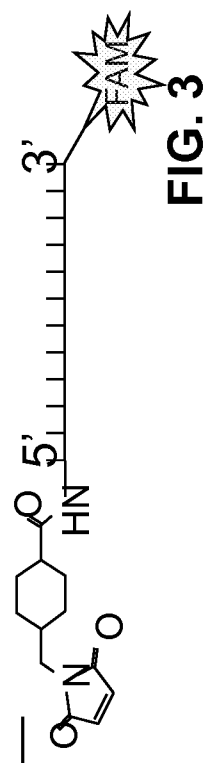
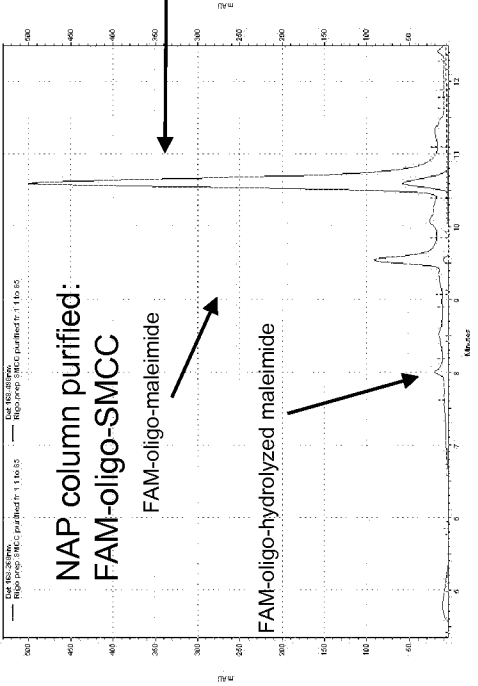
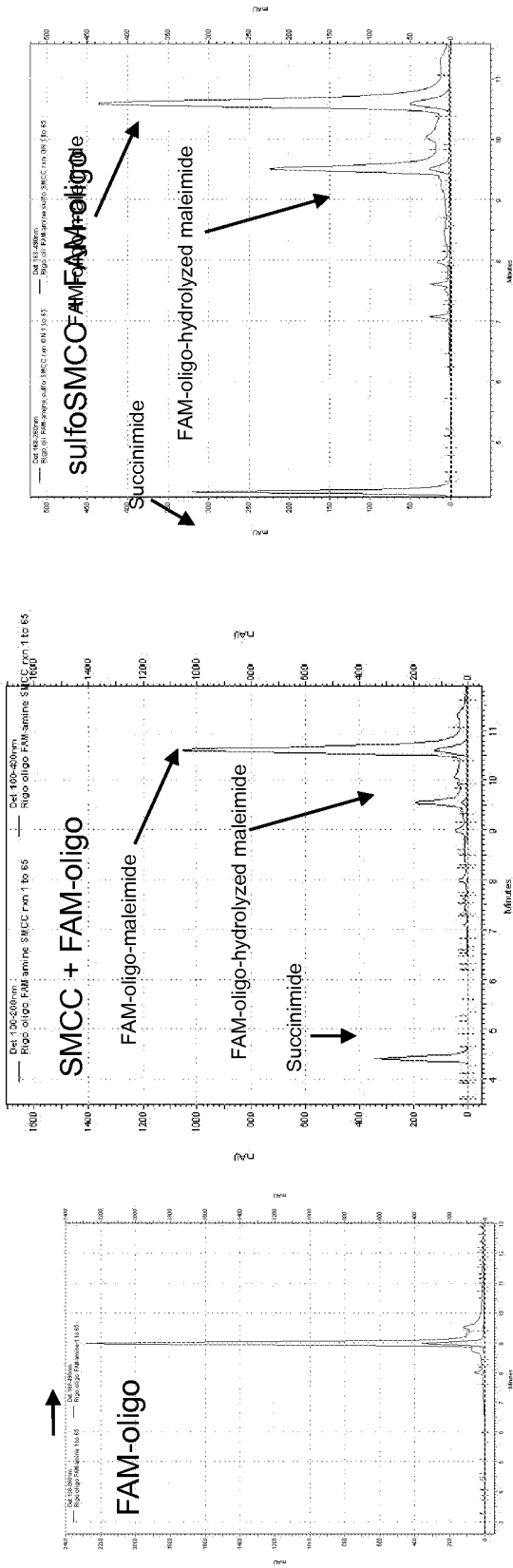


FIG. 3

Tethering reaction

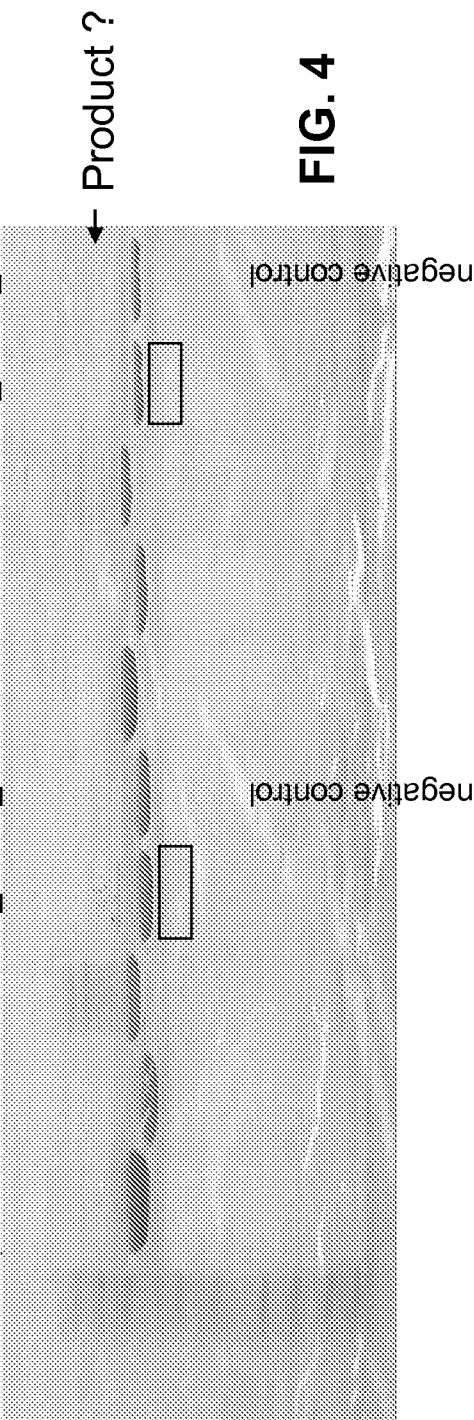
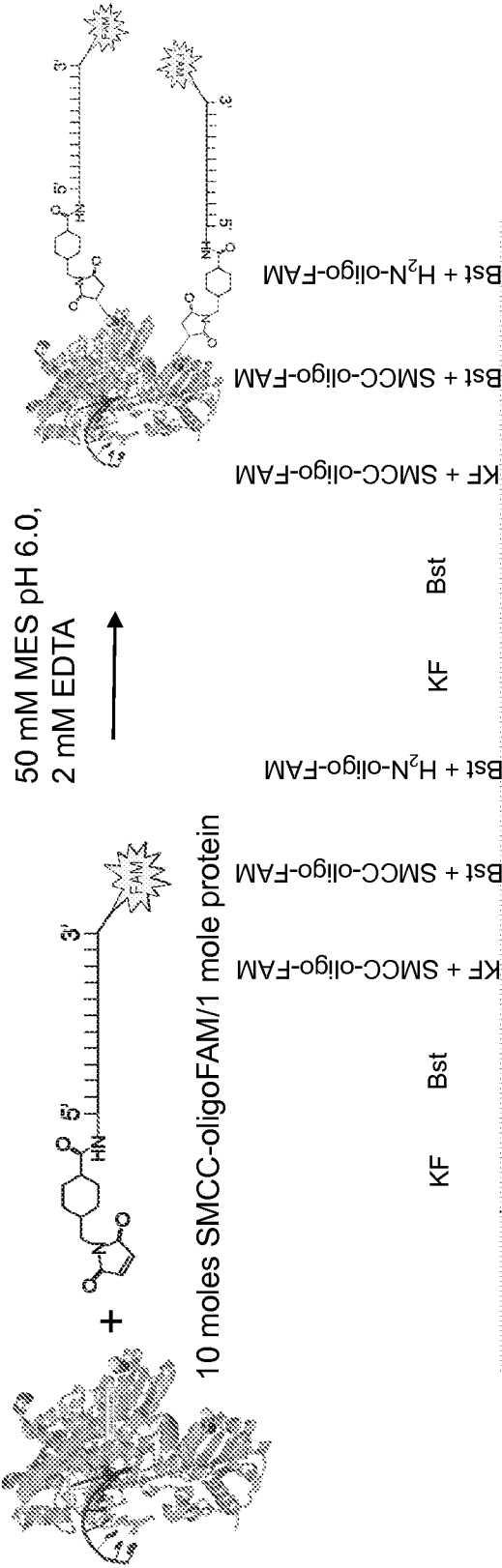


FIG. 4

Polymerase DTT treatment and purification

Purpose: to reduce disulfide bonds

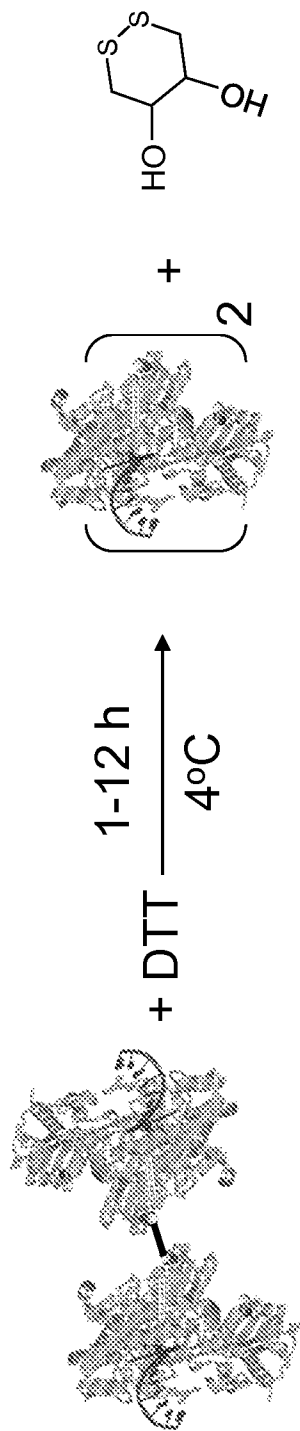


FIG. 5

FAM-oligo-NH₂ activation with SMCC

Purpose: to

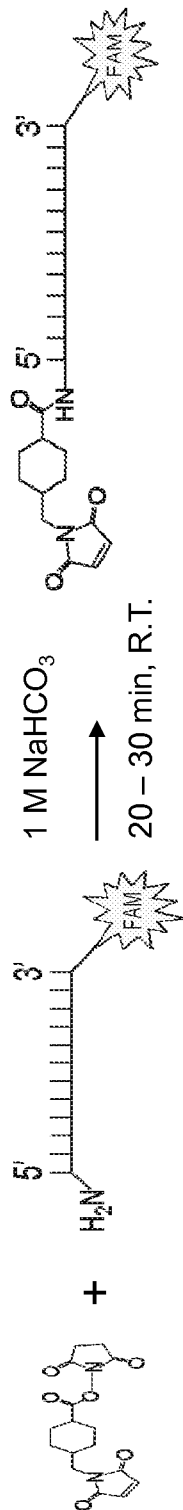


FIG. 6

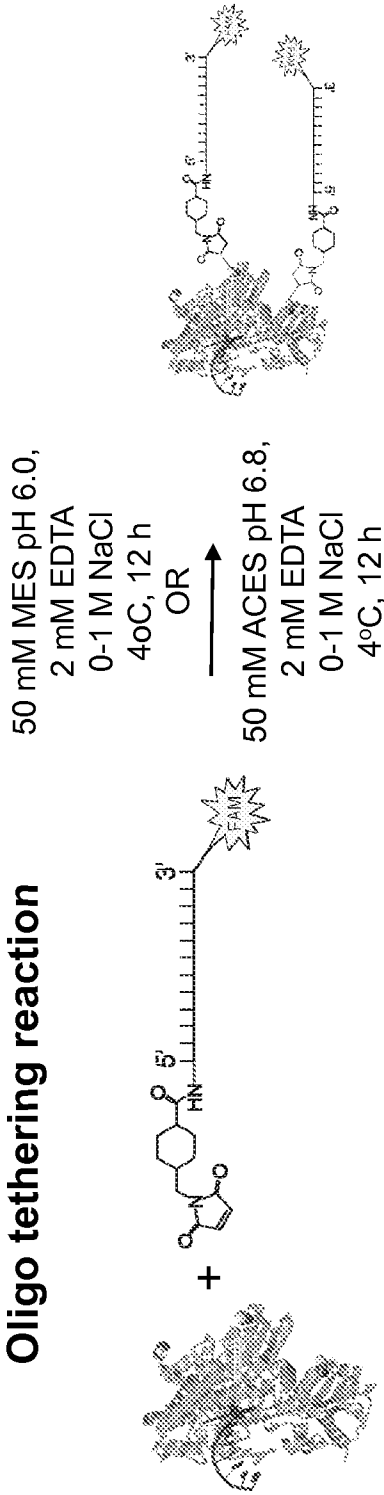


FIG. 7

Oligo tethering reaction results: NaCl pushed reaction yield to greater than 50%

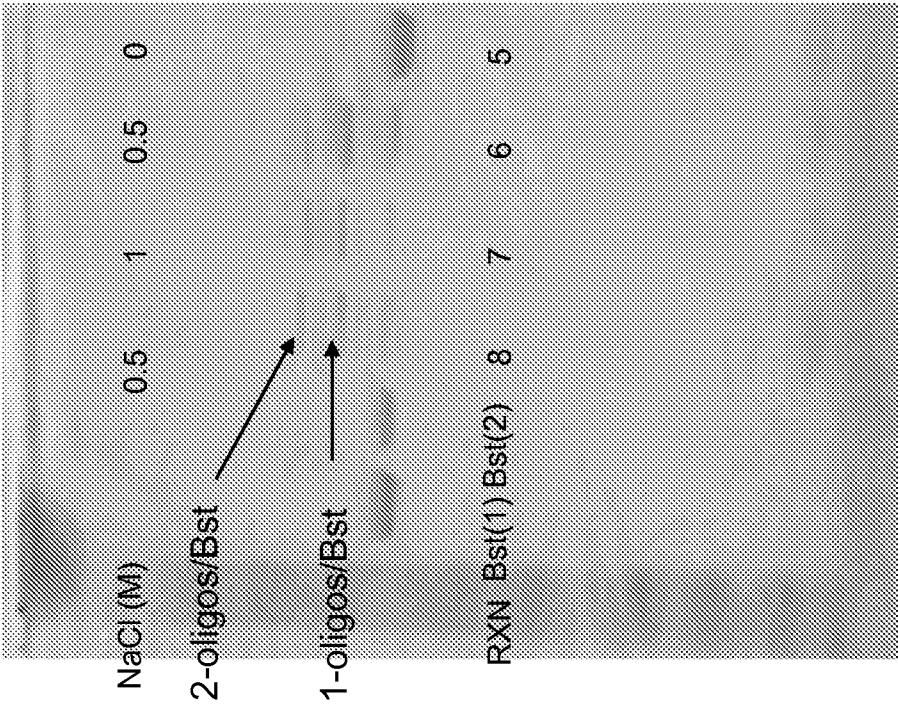


FIG. 8 FAM-oligo-maleimide dissolved in 50 mM ACES pH 6.8, 2 mM EDTA

RXN	Bst (41.6 μM)	FAMoligo- maleimide (264 μM)	NaCl (5M)	Buffer added for gel
RP12092010R5	1 μL	24 μL	0 μL	2.5 μL
RP12092010R6	1 μL	24 μL	2.5 μL	0 μL
RP12092010R7	1 μL	24 μL	5 μL	24 μL
RP12092010R8	1 μL	48 μL	5 μL	0
Bst (1)	1 μL	0 μL		26.5 μL
Bst (2)	1 μL	0 μL		53 μL

High salt washes off untethered polymerase

- Ion Sequencing Pol result zero reads, no signal

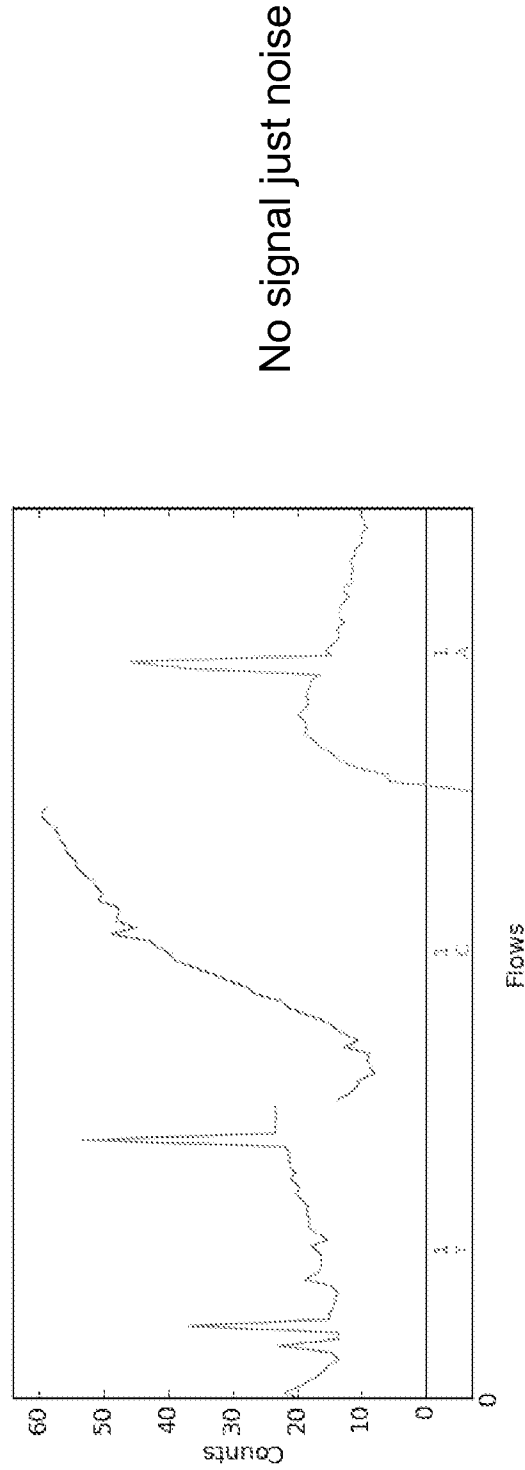


FIG. 9

Tethered polymerase survives high salt washes and produces key signal

- Tethered Ion Sequencing Pol result >800k Q17 reads, key signal>30

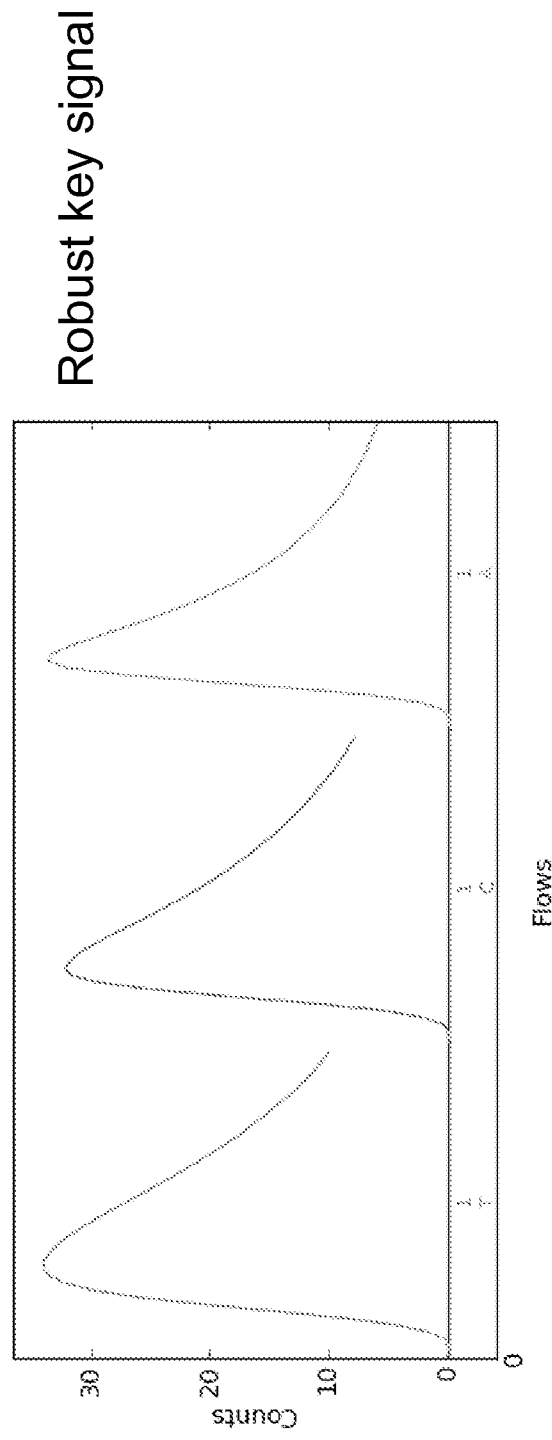


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/024266

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/535 G01N33/543 G01N33/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/016937 A2 (ION TORRENT SYSTEMS INC [US]; ROTHBERG JONATHAN M [US]; LEAMON JOHN H) 11 February 2010 (2010-02-11) paragraph [0164]; figure 8a -----	1-8
X	US 2007/172866 A1 (HARDIN SUSAN [US] ET AL) 26 July 2007 (2007-07-26) paragraph [0064] paragraph [0080] -----	1-8
X	WO 2005/094441 A2 (UNIV CALIFORNIA [US]; WEISS SHIMON [US]; WU ANNA M [US]; PERRY L JEAN) 13 October 2005 (2005-10-13) paragraph [0067]; figures 12, 13 ----- -/-	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 May 2012

Date of mailing of the international search report

25/05/2012

Name and mailing address of the ISA/

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Authorized officer

Routledge, Brian

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/024266

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/042633 A1 (WILLIAMS JOHN G K [US]) 24 February 2005 (2005-02-24) claims paragraph [0054] - paragraph [0055]; figure 2 -----	1-8
X	WO 02/04680 A2 (VISIGEN BIOTECHNOLOGIES INC [US]) 17 January 2002 (2002-01-17) page 17, paragraph final -----	1-8
X	WO 98/25146 A1 (GLAXO GROUP LTD [GB]; DOWER WILLIAM JAMES [US]; HEINKEL GREGORY LYNN []) 11 June 1998 (1998-06-11) claims page 52, line 4 - line 13 -----	1-8
X	US 5 652 107 A (LIZARDI PAUL M [MX] ET AL) 29 July 1997 (1997-07-29) claims figures -----	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/024266

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐

 on paper
 - ☒

 in electronic form
 - b. (time)

☐

 in the international application as filed
 - ☐

 together with the international application in electronic form
 - ☒

 subsequently to this Authority for the purpose of search
2.

☒

 In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2012/024266

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010016937 A2	11-02-2010	EP 2321434 A2	18-05-2011
		US 2010035252 A1	11-02-2010
		US 2011250603 A1	13-10-2011
		US 2011250700 A1	13-10-2011
		US 2011251078 A1	13-10-2011
		US 2011251110 A1	13-10-2011
		WO 2010016937 A2	11-02-2010

US 2007172866 A1	26-07-2007	NONE	

WO 2005094441 A2	13-10-2005	US 2008248463 A1	09-10-2008
		WO 2005094441 A2	13-10-2005

US 2005042633 A1	24-02-2005	US 2005042633 A1	24-02-2005
		US 2009068655 A1	12-03-2009
		US 2009092970 A1	09-04-2009

WO 0204680 A2	17-01-2002	AT 377093 T	15-11-2007
		AU 8288101 A	21-01-2002
		CA 2415897 A1	17-01-2002
		CN 1553953 A	08-12-2004
		CN 101525660 A	09-09-2009
		DE 60131194 T2	07-08-2008
		EP 1368460 A2	10-12-2003
		EP 1975251 A2	01-10-2008
		EP 2100971 A2	16-09-2009
		JP 2004513619 A	13-05-2004
		JP 2009118847 A	04-06-2009
		JP 2009240318 A	22-10-2009
		US 2003064366 A1	03-04-2003
		US 2005260614 A1	24-11-2005
		US 2005266424 A1	01-12-2005
		US 2007172858 A1	26-07-2007
		US 2007172861 A1	26-07-2007
		US 2007172862 A1	26-07-2007
		US 2007172863 A1	26-07-2007
		US 2007172864 A1	26-07-2007
		US 2007172865 A1	26-07-2007
		US 2007172867 A1	26-07-2007
		US 2007172868 A1	26-07-2007
		US 2007184475 A1	09-08-2007
		US 2007275395 A1	29-11-2007
		US 2007292867 A1	20-12-2007
		US 2009275036 A1	05-11-2009
		US 2009305278 A1	10-12-2009
		US 2010255463 A1	07-10-2010
		US 2010304367 A1	02-12-2010
		US 2010317005 A1	16-12-2010
		US 2011014604 A1	20-01-2011
		US 2011021383 A1	27-01-2011
		US 2011059436 A1	10-03-2011
		WO 0204680 A2	17-01-2002

WO 9825146 A1	11-06-1998	AU 5130398 A	29-06-1998
		EP 0944836 A1	29-09-1999
		US 5958703 A	28-09-1999
		US 6309842 B1	30-10-2001
		WO 9825146 A1	11-06-1998

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2012/024266

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5652107	A	29-07-1997	NONE