

TRANSITION-METAL CATALYST COMPOSITIONS AND METHODS FOR SEQUENCING BY SYNTHESIS

BACKGROUND

Field

[0001] The present disclosure generally relates to polynucleotide sequencing methods, compositions, and kits for sequencing. The present disclosure also relates to methods to remove blocking groups.

Description of the Related Art

[0002] Advances in the study of molecules have been led, in part, by improvement in technologies used to characterize the molecules or their biological reactions. In particular, the study of the nucleic acids DNA and RNA has benefited from developing technologies used for sequence analysis and the study of hybridization events.

[0003] An example of the technologies that have improved the study of nucleic acids is the development of fabricated arrays of immobilized nucleic acids. These arrays consist typically of a high-density matrix of polynucleotides immobilized onto a solid support material. See, e.g., Fodor et al., *Trends Biotech.* 12: 19-26, 1994, which describes ways of assembling the nucleic acids using a chemically sensitized glass surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotide phosphoramidites. Fabricated arrays can also be manufactured by the technique of “spotting” known polynucleotides onto a solid support at predetermined positions (e.g., Stimpson et al., *Proc. Natl. Acad. Sci.* 92: 6379-6383, 1995).

[0004] One way of determining the nucleotide sequence of a nucleic acid bound to an array is called “sequencing by synthesis” or “SBS.” This technique for determining the sequence of DNA ideally requires the controlled (i.e., one at a time) incorporation of the correct complementary nucleotide opposite the nucleic acid being sequenced. This allows for accurate sequencing by adding nucleotides in multiple cycles as each nucleotide residue is sequenced one at a time, thus preventing an uncontrolled series of incorporations from occurring. The incorporated nucleotide is read using an appropriate label attached thereto before removal of the label moiety and the subsequent next round of sequencing.

[0005] In order to ensure that only a single incorporation occurs, a structural modification (“protecting group” or “blocking group”) is included in each labeled nucleotide that is added to the growing chain to ensure that only one nucleotide is incorporated. After the nucleotide with the protecting group has been added, the protecting group is then removed, under reaction conditions which do not interfere with the integrity of the DNA being sequenced. The

sequencing cycle can then continue with the incorporation of the next protected, labeled nucleotide. To be useful in DNA sequencing, nucleotides, which are usually nucleotide triphosphates, generally require a 3' hydroxy blocking group so as to prevent the polymerase used to incorporate it into a polynucleotide chain from continuing to replicate once the base on the nucleotide is added.

[0006] Various compositions are employed at each step of a cycle of sequencing. For example, an incorporation composition comprising a polymerase and one or more different types of nucleotides are employed during the incorporation step. A scan composition that may include, among other things, an antioxidant to protect the polynucleotides from photo-induced damage during the detection step when, for example, the nucleotides include fluorophore labels for detection. A deblocking composition that includes reagents for cleaving the blocking moiety (e.g., the 3' hydroxy blocking group) from the nucleotide incorporated is employed during the deblocking step. Cleavage reagents such as palladium (Pd) catalysts prepared from palladium complexes in the presence of water-soluble phosphine ligand(s) has been reported in the deblocking composition, for example, U.S. Publication Nos. 2020/0216891 and 2021/0403500, each of which is incorporated by reference in its entirety. Pd has the capacity to stick on DNA, mostly in its inactive Pd(II) form, which may interfere with the binding between DNA and polymerase, causing increased phasing. A post-cleavage wash composition that includes a Pd scavenger compound may be used following the deblocking step. For example, PCT Publication No. WO 2020/126593 discloses Pd scavengers such as 3,3'-dithiodipropionic acid (DDPA) and lipoic acid (LA) may be included in the scan composition and/or the post-cleavage wash composition. The use of these scavengers in the post-cleave washing solution has the purpose of scavenging Pd(0), converting Pd(0) to the inactive Pd(II) form, thereby improving the prephasing value and sequencing metrics, reducing signal degrade, and extend sequencing read length. However, there exists a continued demand for developing compositions for use at each step of the sequencing to optimize performance.

SUMMARY

[0007] One aspect of the present disclosure relates to a method for determining sequences of a plurality of target polynucleotides, comprising:

- (a) contacting a solid support with sequencing primers under hybridization conditions, wherein the solid support comprises a plurality of target polynucleotides immobilized thereon; and the sequencing primers are complementary to at least a portion of the target polynucleotides;

(b) contacting the solid support with a first aqueous solution comprising DNA polymerase and one or more of four different types of nucleotides under conditions suitable for DNA polymerase-mediated primer extension, wherein each of the nucleotides comprises a 3' blocking group comprising an unsubstituted or substituted allyl group;

(c) incorporating one type of nucleotides into the sequencing primers to produce extended copy polynucleotides;

(d) performing one or more fluorescent measurements of the extended copy polynucleotides;

(e) removing the 3' blocking group of the incorporated nucleotides in a second aqueous solution comprising a transition metal catalyst and one or more reducing agents, the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand, and wherein the one or more reducing agents comprise a boron-containing reducing agent; and

(f) washing the solid support with a third aqueous solution after the removal of the 3' blocking group of the incorporated nucleotides.

[0008] Another aspect of the present disclosure relates to a kit for use with a sequencing apparatus, comprising: an aqueous cleavage mixture comprising a transition metal catalyst and one or more reducing agents, wherein the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand, and wherein the one or more reducing agents comprise a boron-containing reducing agent.

[0009] A further aspect of the present disclosure relates to a cartridge for use with a sequencing apparatus, comprising a plurality of chambers, wherein one of the plurality of chambers is for use with the kit comprising the aqueous cleavage mixture described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **FIG. 1** is a line chart of percent cleavage of the 3' blocking group as a function of time for a Pd(II) complex mixed with a reducing phosphine ligand (THPP), a non-reducing phosphine ligand (TCEP) without a reducing agent, and a non-reducing phosphine ligand (TCEP) with a reducing agent (NH_3BH_3).

[0011] **FIG. 2** is a line chart of percent cleavage of the 3' blocking group as a function of time when a Pd(II) complex mixed with a non-reducing phosphine ligand TCEP in the presence of a reducing agent at pH 7, in comparison to a standard universal cleavage mixture (UCM) at two different pH (pH of 7 and 9.5).

[0012] **FIG. 3** is a line chart of percent cleavage of the 3' blocking group as a function of time when for a Pd(II) complex mixed with TCEP at 50% concentration, when compared to a standard UCM at 100% or 50% palladium catalyst concentration.

[0013] **FIG. 4** is a line chart of percent phasing and percent prephasing as a function of sequencing cycles on Illumina's iSeq™ platform, comparing the performance of a standard UCM with a Pd cleavage mixture according to an embodiment of the present disclosure.

[0014] **FIG. 5A** illustrates the Pd(0) formation using a standard universal cleavage mixture in comparison to a palladium catalyst generated from a N,N-bidentate non-phosphine ligand.

[0015] **FIG. 5B** illustrates the percent cleavage of a substrate containing an AOM group using a standard universal cleavage mixture in comparison to a palladium catalyst generated from a N,N-bidentate non-phosphine ligand.

DETAILED DESCRIPTION

[0016] Some aspects of the present disclosure relate to methods of nucleic acid sequencing where transition metal catalyst activation includes separate coordination and reduction steps. In particular, the sequencing method described herein involves the use of a transition metal catalyst, for example a palladium (Pd) catalyst or a nickel (Ni) catalyst, with a non-reducing ligand, for example a water-soluble phosphine, and a reducing agent to cleave the 3' hydroxy blocking group of an incorporated nucleotide prior to the next incorporation cycle. In some embodiments, the water-soluble phosphine includes a tris[substituted alkyl] phosphine. In some embodiments, the palladium (Pd) complex is a Pd(0) complex. In some embodiments, the nickel (Ni) complex is a Ni(0) complex. In some embodiments, the reducing agent includes boron. Some aspects of the present disclosure relate to methods of removing a blocking group generally.

Definitions

[0017] 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. The use of the term "including" as well as other forms, such as "include," "includes," and "included," is not limiting. The use of the term "having" as well as other forms, such as "have," "has," and "had," is not limiting. As used in this specification, whether in a transitional phrase or in the body of the claim, the terms "comprise(s)" and "comprising" are to be interpreted as having an open-ended meaning. That is, the above terms are to be interpreted synonymously with the phrases "having at least" or "including at least." For example, when used in the context of a process, the term "comprising" means that the process includes at least the recited steps, but may include additional

steps. When used in the context of a compound, composition, or device, the term “comprising” means that the compound, composition, or device includes at least the recited features or components, but may also include additional features or components.

[0018] Where a range of values is provided, it is understood that the upper and lower limit, and each intervening value between the upper and lower limit of the range is encompassed within the embodiments.

[0019] As used herein, common organic abbreviations are defined as follows:

°C	Temperature in degrees Centigrade
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ddNTP	Dideoxynucleotide triphosphate
ffN	Fully functionalized nucleotide
ffA	Fully functionalized “A” nucleotide
ffC	Fully functionalized “C” nucleotide
ffT	Fully functionalized “T” nucleotide
ffG	Fully functionalized “G” nucleotide
IMX	Incorporation mix or Incorporation mixture
Ni	Nickel
Pd	Palladium
RT	Room temperature
SBS	Sequencing by Synthesis
TCEP	tris(2-carboxethyl)phosphine
THPP	tris(hydroxypropyl)phosphine

[0020] As used herein, the term “array” refers to a population of different probe molecules that are attached to one or more substrates such that the different probe molecules can be differentiated from each other according to relative location. An array can include different probe molecules that are each located at a different addressable location on a substrate. Alternatively, or additionally, an array can include separate substrates each bearing a different probe molecule, wherein the different probe molecules can be identified according to the locations of the substrates on a surface to which the substrates are attached or according to the locations of the substrates in a liquid. Exemplary arrays in which separate substrates are located on a surface include, without limitation, those including beads in wells as described, for example, in U.S. Patent No. 6,355,431 B1, US 2002/0102578 and PCT Publication No. WO 00/63437. Exemplary

formats that can be used in the invention to distinguish beads in a liquid array, for example, using a microfluidic device, such as a fluorescent activated cell sorter (FACS), are described, for example, in US Pat. No. 6,524,793. Further examples of arrays that can be used in the invention include, without limitation, those described in U.S. Pat Nos. 5,429,807; 5,436,327; 5,561,071; 5,583,211; 5,658,734; 5,837,858; 5,874,219; 5,919,523; 6,136,269; 6,287,768; 6,287,776; 6,288,220; 6,297,006; 6,291,193; 6,346,413; 6,416,949; 6,482,591; 6,514,751 and 6,610,482; and WO 93/17126; WO 95/11995; WO 95/35505; EP 742 287; and EP 799 897.

[0021] As used herein, the term “covalently attached” or “covalently bonded” refers to the forming of a chemical bonding that is characterized by the sharing of pairs of electrons between atoms. For example, a covalently attached polymer coating refers to a polymer coating that forms chemical bonds with a functionalized surface of a substrate, as compared to attachment to the surface via other means, for example, adhesion or electrostatic interaction. It will be appreciated that polymers that are attached covalently to a surface can also be bonded via means in addition to covalent attachment.

[0022] As used herein, “activate” or “activating” a transition metal catalyst (e.g. a palladium catalyst or a nickel catalyst) include but are not limited to (1) exposing the transition metal in an inactive form (e.g. Pd(II) or Ni(II)) to a reducing ligand to convert the transition metal into an active form (e.g. Pd(0) or Ni(0)), or (2) exposing the transition metal in an inactive form (e.g. Pd(II) or Ni(II)) to a non-reducing ligand and a reducing agent to convert the transition metal into an active form (e.g. Pd(0) or Ni(0)).

[0023] As used herein, “inactivate” or “inactivating” a palladium catalyst include but are not limited to the following several mechanisms of using a palladium scavenger: (1) the palladium scavenger may act as a competitive substrate to consume any residual active Pd(0) sticking on the nucleic acid; (2) the palladium scavenger may act as an oxidizer to convert the active Pd(0) to the inactive Pd(II) form; and (3) the palladium scavenger may act as a competitive ligand to remove the Pd (e.g., Pd(0) or Pd(II)) sticking on the nucleic acid.

[0024] As used herein, any “R” group(s) represent substituents that can be attached to the indicated atom. An R group may be substituted or unsubstituted.

[0025] It is to be understood that certain radical naming conventions can include either a mono-radical or a di-radical, depending on the context. For example, where a substituent requires two points of attachment to the rest of the molecule, it is understood that the substituent is a di-radical. For example, a substituent identified as alkyl that requires two points of attachment includes di-radicals such as $-\text{CH}_2-$, $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2-$, and the like. Other radical naming conventions clearly indicate that the radical is a di-radical such as “alkylene” or “alkenylene.”

[0026] The term “halogen” or “halo,” as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, *e.g.*, fluorine, chlorine, bromine, or iodine, with fluorine and chlorine being preferred.

[0027] As used herein, “C_a to C_b,” “C_a-C_b,” or “C_{a-b}” in which “a” and “b” are integers refer to the number of carbon atoms in an alkyl, alkenyl or alkynyl group, or the number of ring atoms of a cycloalkyl or aryl group. That is, the alkyl, the alkenyl, the alkynyl, the ring of the cycloalkyl, and ring of the aryl can contain from “a” to “b,” inclusive, carbon atoms. For example, a “C₁ to C₄ alkyl” group refers to all alkyl groups having from 1 to 4 carbons, that is, CH₃-, CH₃CH₂-, CH₃CH₂CH₂-, (CH₃)₂CH-, CH₃CH₂CH₂CH₂-, CH₃CH₂CH(CH₃)- and (CH₃)₃C-; a C₃ to C₄ cycloalkyl group refers to all cycloalkyl groups having from 3 to 4 carbon atoms, that is, cyclopropyl and cyclobutyl. Similarly, a “4 to 6 membered heterocyclyl” group refers to all heterocyclyl groups with 4 to 6 total ring atoms, for example, azetidine, oxetane, oxazoline, pyrrolidine, piperidine, piperazine, morpholine, and the like. If no “a” and “b” are designated with regard to an alkyl, alkenyl, alkynyl, cycloalkyl, or aryl group, the broadest range described in these definitions is to be assumed. As used herein, the term “C₁-C₆” includes C₁, C₂, C₃, C₄, C₅ and C₆, and a range defined by any of the two numbers. For example, C₁-C₆ alkyl includes C₁, C₂, C₃, C₄, C₅ and C₆ alkyl, C₂-C₆ alkyl, C₁-C₃ alkyl, etc. Similarly, C₂-C₆ alkenyl includes C₂, C₃, C₄, C₅ and C₆ alkenyl, C₂-C₅ alkenyl, C₃-C₄ alkenyl, etc.; and C₂-C₆ alkynyl includes C₂, C₃, C₄, C₅ and C₆ alkynyl, C₂-C₅ alkynyl, C₃-C₄ alkynyl, etc. C₃-C₈ cycloalkyl each includes hydrocarbon ring containing 3, 4, 5, 6, 7 and 8 carbon atoms, or a range defined by any of the two numbers, such as C₃-C₇ cycloalkyl or C₅-C₆ cycloalkyl.

[0028] As used herein, “alkyl” refers to a straight or branched hydrocarbon chain that is fully saturated (*i.e.*, contains no double or triple bonds). The alkyl group may have 1 to 20 carbon atoms (whenever it appears herein, a numerical range such as “1 to 20” refers to each integer in the given range; *e.g.*, “1 to 20 carbon atoms” means that the alkyl group may consist of 1 carbon atom, 2 carbon atoms, 3 carbon atoms, *etc.*, up to and including 20 carbon atoms, although the present definition also covers the occurrence of the term “alkyl” where no numerical range is designated). The alkyl group may also be a medium size alkyl having 1 to 9 carbon atoms. The alkyl group could also be a lower alkyl having 1 to 6 carbon atoms. The alkyl group may be designated as “C₁-C₄ alkyl” or similar designations. By way of example only, “C₁-C₆ alkyl” indicates that there are one to six carbon atoms in the alkyl chain, *i.e.*, the alkyl chain is selected from the group consisting of methyl, ethyl, propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, and t-butyl. Typical alkyl groups include, but are in no way limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, pentyl, hexyl, and the like.

[0029] As used herein, “alkoxy” refers to the formula –OR wherein R is an alkyl as is defined above, such as “C₁-C₉ alkoxy,” including but not limited to methoxy, ethoxy, n-propoxy, 1-methylethoxy (isopropoxy), n-butoxy, iso-butoxy, sec-butoxy, and tert-butoxy, and the like.

[0030] As used herein, “alkenyl” refers to a straight or branched hydrocarbon chain containing one or more double bonds. The alkenyl group may have 2 to 20 carbon atoms, although the present definition also covers the occurrence of the term “alkenyl” where no numerical range is designated. The alkenyl group may also be a medium size alkenyl having 2 to 9 carbon atoms. The alkenyl group could also be a lower alkenyl having 2 to 6 carbon atoms. The alkenyl group may be designated as “C₂-C₆ alkenyl” or similar designations. By way of example only, “C₂-C₆ alkenyl” indicates that there are two to six carbon atoms in the alkenyl chain, i.e., the alkenyl chain is selected from the group consisting of ethenyl, propen-1-yl, propen-2-yl, propen-3-yl, buten-1-yl, buten-2-yl, buten-3-yl, buten-4-yl, 1-methyl-propen-1-yl, 2-methyl-propen-1-yl, 1-ethyl-ethen-1-yl, 2-methyl-propen-3-yl, buta-1,3-dienyl, buta-1,2,-dienyl, and buta-1,2-dien-4-yl. Typical alkenyl groups include, but are in no way limited to, ethenyl, propenyl, butenyl, pentenyl, and hexenyl, and the like.

[0031] The term “aromatic” refers to a ring or ring system having a conjugated pi electron system and includes both carbocyclic aromatic (e.g., phenyl) and heterocyclic aromatic groups (e.g., pyridine). The term includes monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of atoms) groups provided that the entire ring system is aromatic.

[0032] As used herein, “aryl” refers to an aromatic ring or ring system (i.e., two or more fused rings that share two adjacent carbon atoms) containing only carbon in the ring backbone. When the aryl is a ring system, every ring in the system is aromatic. The aryl group may have 6 to 18 carbon atoms, although the present definition also covers the occurrence of the term “aryl” where no numerical range is designated. In some embodiments, the aryl group has 6 to 10 carbon atoms. The aryl group may be designated as “C₆-C₁₀ aryl,” “C₆ or C₁₀ aryl,” or similar designations. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, azulenyl, and anthracenyl.

[0033] An “aralkyl” or “arylalkyl” is an aryl group connected, as a substituent, via an alkylene group, such as “C₇₋₁₄ aralkyl” and the like, including but not limited to benzyl, 2-phenylethyl, 3-phenylpropyl, and naphthylalkyl. In some cases, the alkylene group is a lower alkylene group (i.e., a C₁-C₆ alkylene group).

[0034] As used herein, “aryloxy” refers to RO- in which R is an aryl, as defined above, such as but not limited to phenyl.

[0035] As used herein, “heteroaryl” refers to an aromatic ring or ring system (i.e., two or more fused rings that share two adjacent atoms) that contain(s) one or more heteroatoms, that

is, an element other than carbon, including but not limited to, nitrogen, oxygen and sulfur, in the ring backbone. When the heteroaryl is a ring system, every ring in the system is aromatic. The heteroaryl group may have 5-18 ring members (i.e., the number of atoms making up the ring backbone, including carbon atoms and heteroatoms), although the present definition also covers the occurrence of the term “heteroaryl” where no numerical range is designated. In some embodiments, the heteroaryl group has 5 to 10 ring members or 5 to 7 ring members. The heteroaryl group may be designated as “5-7 membered heteroaryl,” “5-10 membered heteroaryl,” or similar designations. Examples of heteroaryl rings include, but are not limited to, furyl, thienyl, phthalazinyl, pyrrolyl, oxazolyl, thiazolyl, imidazolyl, pyrazolyl, isoxazolyl, isothiazolyl, triazolyl, thiadiazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl, quinolinyl, isoquinolinyl, benzoimidazolyl, benzoxazolyl, benzothiazolyl, indolyl, isoindolyl, and benzothienyl.

[0036] A “heteroaralkyl” or “heteroarylalkyl” is heteroaryl group connected, as a substituent, via an alkylene group. Examples include but are not limited to 2-thienylmethyl, 3-thienylmethyl, furylmethyl, thienylethyl, pyrrolylalkyl, pyridylalkyl, isoxazolylalkyl, and imidazolylalkyl. In some cases, the alkylene group is a lower alkylene group (i.e., a C₁-C₆ alkylene group).

[0037] As used herein, “carbocyclyl” means a non-aromatic cyclic ring or ring system containing only carbon atoms in the ring system backbone. When the carbocyclyl is a ring system, two or more rings may be joined together in a fused, bridged or spiro-connected fashion. Carbocyclyls may have any degree of saturation provided that at least one ring in a ring system is not aromatic. Thus, carbocyclyls include cycloalkyls, cycloalkenyls, and cycloalkynyls. The carbocyclyl group may have 3 to 20 carbon atoms, although the present definition also covers the occurrence of the term “carbocyclyl” where no numerical range is designated. The carbocyclyl group may also be a medium size carbocyclyl having 3 to 10 carbon atoms. The carbocyclyl group could also be a carbocyclyl having 3 to 6 carbon atoms. The carbocyclyl group may be designated as “C₃-C₆ carbocyclyl” or similar designations. Examples of carbocyclyl rings include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclohexenyl, 2,3-dihydro-indene, bicycle[2.2.2]octanyl, adamantyl, and spiro[4.4]nonanyl.

[0038] As used herein, “cycloalkyl” means a fully saturated carbocyclyl ring or ring system. Examples include cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

[0039] As used herein, “heterocyclyl” means a non-aromatic cyclic ring or ring system containing at least one heteroatom in the ring backbone. Heterocyclyls may be joined together in a fused, bridged or spiro-connected fashion. Heterocyclyls may have any degree of saturation provided that at least one ring in the ring system is not aromatic. The heteroatom(s) may be

present in either a non-aromatic or aromatic ring in the ring system. The heterocyclyl group may have 3 to 20 ring members (i.e., the number of atoms making up the ring backbone, including carbon atoms and heteroatoms), although the present definition also covers the occurrence of the term “heterocyclyl” where no numerical range is designated. The heterocyclyl group may also be a medium size heterocyclyl having 3 to 10 ring members. The heterocyclyl group could also be a heterocyclyl having 3 to 6 ring members. The heterocyclyl group may be designated as “3-6 membered heterocyclyl” or similar designations. In preferred six membered monocyclic heterocyclyls, the heteroatom(s) are selected from one up to three of O, N or S, and in preferred five membered monocyclic heterocyclyls, the heteroatom(s) are selected from one or two heteroatoms selected from O, N, or S. Examples of heterocyclyl rings include, but are not limited to, azepinyl, acridinyl, carbazolyl, cinnolinyl, dioxolanyl, imidazolanyl, imidazolidinyl, morpholinyl, oxiranyl, oxepanyl, thiepanyl, piperidinyl, piperazinyl, dioxopiperazinyl, pyrrolidinyl, pyrrolidonyl, pyrrolidionyl, 4-piperidonyl, pyrazolinyl, pyrazolidinyl, 1,3-dioxinyl, 1,3-dioxanyl, 1,4-dioxinyl, 1,4-dioxanyl, 1,3-oxathianyl, 1,4-oxathiinyl, 1,4-oxathianyl, 2*H*-1,2-oxazinyl, trioxanyl, hexahydro-1,3,5-triazinyl, 1,3-dioxolyl, 1,3-dioxolanyl, 1,3-dithiolyl, 1,3-dithiolanyl, isoxazolanyl, isoxazolidinyl, oxazolanyl, oxazolidinyl, oxazolidinonyl, thiazolanyl, thiazolidinyl, 1,3-oxathiolanyl, indolanyl, isoindolanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydro-1,4-thiazinyl, thiamorpholinyl, dihydrobenzofuranyl, benzimidazolidinyl, and tetrahydroquinoline.

[0040] As used herein, “(aryl)alkyl” refer to an aryl group, as defined above, connected, as a substituent, via an alkylene group, as described above. The alkylene and aryl group of an aralkyl may be substituted or unsubstituted. Examples include but are not limited to benzyl, 2-phenylalkyl, 3-phenylalkyl, and naphthylalkyl. In some embodiments, the alkylene is an unsubstituted straight chain containing 1, 2, 3, 4, 5, or 6 methylene unit(s).

[0041] As used herein, “(heteroaryl)alkyl” refer to a heteroaryl group, as defined above, connected, as a substituent, via an alkylene group, as defined above. The alkylene and heteroaryl group of heteroaralkyl may be substituted or unsubstituted. Examples include but are not limited to 2-thienylalkyl, 3-thienylalkyl, furylalkyl, thienylalkyl, pyrrolylalkyl, pyridylalkyl, isoxazolylalkyl, and imidazolylalkyl, and their benzo-fused analogs. In some embodiments, the alkylene is an unsubstituted straight chain containing 1, 2, 3, 4, 5, or 6 methylene unit(s).

[0042] As used herein, “(heterocyclyl)alkyl” refer to a heterocyclic or a heterocyclyl group, as defined above, connected, as a substituent, via an alkylene group, as defined above. The alkylene and heterocyclyl groups of a (heterocyclyl)alkyl may be substituted or unsubstituted. Examples include but are not limited to (tetrahydro-2*H*-pyran-4-yl)methyl, (piperidin-4-yl)ethyl, (piperidin-4-yl)propyl, (tetrahydro-2*H*-thiopyran-4-yl)methyl, and (1,3-thiazinan-4-yl)methyl. In

some embodiments, the alkylene is an unsubstituted straight chain containing 1, 2, 3, 4, 5, or 6 methylene unit(s).

[0043] As used herein, “(carbocyclyl)alkyl” refer to a carbocyclyl group (as defined herein) connected, as a substituent, via an alkylene group. Examples include but are not limited to cyclopropylmethyl, cyclobutylmethyl, cyclopentylethyl, and cyclohexylpropyl. In some embodiments, the alkylene is an unsubstituted straight chain containing 1, 2, 3, 4, 5, or 6 methylene unit(s).

[0044] As used herein, “alkoxyalkyl” or “(alkoxy)alkyl” refers to an alkoxy group connected via an alkylene group, such as C₂-C₈ alkoxyalkyl, or (C₁-C₆ alkoxy)C₁-C₆ alkyl, for example, $-(CH_2)_{1-3}-OCH_3$.

[0045] As used herein, “-O-alkoxyalkyl” or “-O-(alkoxy)alkyl” refers to an alkoxy group connected via an -O-(alkylene) group, such as -O-(C₁-C₆ alkoxy)C₁-C₆ alkyl, for example, $-O-(CH_2)_{1-3}-OCH_3$.

[0046] As used herein, “haloalkyl” refers to an alkyl group in which one or more of the hydrogen atoms are replaced by a halogen (*e.g.*, mono-haloalkyl, di-haloalkyl, and tri-haloalkyl). Such groups include but are not limited to, chloromethyl, fluoromethyl, difluoromethyl, trifluoromethyl and 1-chloro-2-fluoromethyl, 2-fluoroisobutyl. A haloalkyl may be substituted or unsubstituted.

[0047] As used herein, “haloalkoxy” refers to an alkoxy group in which one or more of the hydrogen atoms are replaced by a halogen (*e.g.*, mono-haloalkoxy, di-haloalkoxy and tri-haloalkoxy). Such groups include but are not limited to, chloromethoxy, fluoromethoxy, difluoromethoxy, trifluoromethoxy and 1-chloro-2-fluoromethoxy, 2-fluoroisobutoxy. A haloalkoxy may be substituted or unsubstituted.

[0048] An “amino” group refers to a -NH₂ group. The term “mono-substituted amino group” as used herein refers to an amino (-NH₂) group where one of the hydrogen atom is replaced by a substituent. The term “di-substituted amino group” as used herein refers to an amino (-NH₂) group where each of the two hydrogen atoms is replaced by a substituent. The term “optionally substituted amino,” as used herein refer to a -NR_AR_B group where R_A and R_B are independently hydrogen, alkyl, cycloalkyl, aryl, heteroaryl, heterocyclyl, aralkyl, or heterocyclyl(alkyl), as defined herein.

[0049] An “O-carboxy” group refers to a “-OC(=O)R” group in which R is selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0050] A “C-carboxy” group refers to a “-C(=O)OR” group in which R is selected from the group consisting of hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇

carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein. A non-limiting example includes carboxyl (i.e., -C(=O)OH).

[0051] A “sulfonyl” group refers to an “-SO₂R” group in which R is selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0052] A “sulfinio” group refers to a “-S(=O)OH” group.

[0053] A “sulfo” group refers to a “-S(=O)₂OH” or “-SO₃H” group.

[0054] A “sulfonate” group refers to a “-SO₃⁻” group.

[0055] A “sulfate” group refers to “-SO₄⁻” group.

[0056] A “S-sulfonamido” group refers to a “-SO₂NR_AR_B” group in which R_A and R_B are each independently selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0057] An “N-sulfonamido” group refers to a “-N(R_A)SO₂R_B” group in which R_A and R_B are each independently selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0058] A “C-amido” group refers to a “-C(=O)NR_AR_B” group in which R_A and R_B are each independently selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0059] An “N-amido” group refers to a “-N(R_A)C(=O)R_B” group in which R_A and R_B are each independently selected from hydrogen, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ carbocyclyl, C₆-C₁₀ aryl, 5-10 membered heteroaryl, and 3-10 membered heterocyclyl, as defined herein.

[0060] An “O-carbamyl” group refers to a “-OC(=O)N(R_AR_B)” group in which R_A and R_B can be the same as defined with respect to S-sulfonamido. An O-carbamyl may be substituted or unsubstituted.

[0061] An “N-carbamyl” group refers to an “ROC(=O)N(R_A)” group in which R and R_A can be the same as defined with respect to N-sulfonamido. An N-carbamyl may be substituted or unsubstituted.

[0062] An “O-thiocarbamyl” group refers to a “-OC(=S)-N(R_AR_B)” group in which R_A and R_B can be the same as defined with respect to S-sulfonamido. An O-thiocarbamyl may be substituted or unsubstituted.

[0063] An “N-thiocarbamyl” group refers to an “ROC(=S)N(R_A)-” group in which R and R_A can be the same as defined with respect to N-sulfonamido. An N-thiocarbamyl may be substituted or unsubstituted.

[0064] The term “alkylamino” or “(alkyl)amino” refers to an amino group wherein one or both hydrogen is replaced by an alkyl group.

[0065] An “(alkoxy)alkyl” group refers to an alkoxy group connected via an alkylene group, such as a “(C₁-C₆ alkoxy) C₁-C₆ alkyl” and the like.

[0066] The term “hydroxy” as used herein refers to a -OH group.

[0067] The term “cyano” group as used herein refers to a -CN” group.

[0068] The term “azido” as used herein refers to a -N₃ group.

[0069] When a group is described as “optionally substituted” it may be either unsubstituted or substituted. Likewise, when a group is described as being “substituted,” the substituent may be selected from one or more of the indicated substituents. As used herein, a substituted group is derived from the unsubstituted parent group in which there has been an exchange of one or more hydrogen atoms for another atom or group. Unless otherwise indicated, when a group is deemed to be “substituted,” it is meant that the group is substituted with one or more substituents independently selected from C₁-C₆ alkyl, C₁-C₆ alkenyl, C₁-C₆ alkynyl, C₁-C₆ heteroalkyl, C₃-C₇ carbocyclyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), C₃-C₇carbocyclyl-C₁-C₆-alkyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), 3-10 membered heterocyclyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), 3-10 membered heterocyclyl-C₁-C₆-alkyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), aryl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), (aryl)C₁-C₆ alkyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), 5-10 membered heteroaryl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), (5-10 membered heteroaryl)C₁-C₆ alkyl (optionally substituted with halo, C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, and C₁-C₆ haloalkoxy), halo, -CN, hydroxy, C₁-C₆ alkoxy, (C₁-C₆ alkoxy)C₁-C₆ alkyl, -O(C₁-C₆ alkoxy)C₁-C₆ alkyl; (C₁-C₆ haloalkoxy)C₁-C₆ alkyl; -O(C₁-C₆ haloalkoxy)C₁-C₆ alkyl; aryloxy, sulfhydryl (mercapto), halo(C₁-C₆)alkyl (e.g., -CF₃), halo(C₁-C₆)alkoxy (e.g., -OCF₃), C₁-C₆ alkylthio, arylthio, amino, amino(C₁-C₆)alkyl, nitro, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, S-sulfonamido, N-sulfonamido, C-carboxy, O-carboxy, acyl, cyanato, isocyanato, thiocyanato, isothiocyanato, sulfinyl, sulfonyl, -SO₃H, sulfonate, sulfate, sulfinio, -OSO₂C₁₋₄alkyl, monophosphate, diphosphate, triphosphate, and oxo (=O). Wherever a

group is described as “optionally substituted” that group can be substituted with the above substituents.

[0070] As understood by one of ordinary skill in the art, a compound described herein may exist in ionized form, e.g., -CO_2^- , -SO_3^- or -O-SO_3^- . If a compound contains a positively or negatively charged substituent group, for example, -SO_3^- , it may also contain a negatively or positively charged counterion such that the compound as a whole is neutral. In other aspects, the compound may exist in a salt form, where the counterion is provided by a conjugate acid or base.

[0071] As used herein, a “nucleotide” includes a nitrogen containing heterocyclic base, a sugar, and one or more phosphate groups. They are monomeric units of a nucleic acid sequence. In RNA, the sugar is a ribose, and in DNA a deoxyribose, *i.e.* a sugar lacking a hydroxy group that is present in ribose. The nitrogen containing heterocyclic base can be purine or pyrimidine base. Purine bases include adenine (A) and guanine (G), and modified derivatives or analogs thereof, such as 7-deaza adenine or 7-deaza guanine. Pyrimidine bases include cytosine (C), thymine (T), and uracil (U), and modified derivatives or analogs thereof. The C-1 atom of deoxyribose is bonded to N-1 of a pyrimidine or N-9 of a purine.

[0072] As used herein, a “nucleoside” is structurally similar to a nucleotide, but is missing the phosphate moieties. An example of a nucleoside analogue would be one in which the label is linked to the base and there is no phosphate group attached to the sugar molecule. The term “nucleoside” is used herein in its ordinary sense as understood by those skilled in the art. Examples include, but are not limited to, a ribonucleoside comprising a ribose moiety and a deoxyribonucleoside comprising a deoxyribose moiety. A modified pentose moiety is a pentose moiety in which an oxygen atom has been replaced with a carbon and/or a carbon has been replaced with a sulfur or an oxygen atom. A “nucleoside” is a monomer that can have a substituted base and/or sugar moiety. Additionally, a nucleoside can be incorporated into larger DNA and/or RNA polymers and oligomers.

[0073] The term “purine base” is used herein in its ordinary sense as understood by those skilled in the art, and includes its tautomers. Similarly, the term “pyrimidine base” is used herein in its ordinary sense as understood by those skilled in the art, and includes its tautomers. A non-limiting list of optionally substituted purine-bases includes purine, adenine, guanine, deazapurine, 7-deaza adenine, 7-deaza guanine, hypoxanthine, xanthine, alloxanthine, 7-alkylguanine (e.g., 7-methylguanine), theobromine, caffeine, uric acid and isoguanine. Examples of pyrimidine bases include, but are not limited to, cytosine, thymine, uracil, 5,6-dihydrouracil and 5-alkylcytosine (e.g., 5-methylcytosine).

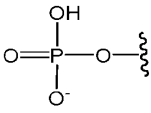
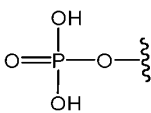
[0074] As used herein, when an oligonucleotide or polynucleotide is described as “comprising” or “incorporating” a nucleoside or nucleotide described herein, it means that the

nucleoside or nucleotide described herein forms a covalent bond with the oligonucleotide or polynucleotide. Similarly, when a nucleoside or nucleotide is described as part of an oligonucleotide or polynucleotide, such as “incorporated into” an oligonucleotide or polynucleotide, it means that the nucleoside or nucleotide described herein forms a covalent bond with the oligonucleotide or polynucleotide. In some such embodiments, the covalent bond is formed between a 3' hydroxy group of the oligonucleotide or polynucleotide with the 5' phosphate group of a nucleotide described herein as a phosphodiester bond between the 3' carbon atom of the oligonucleotide or polynucleotide and the 5' carbon atom of the nucleotide.

[0075] As used herein, the term “cleavable linker” is not meant to imply that the whole linker is required to be removed. The cleavage site can be located at a position on the linker that ensures that part of the linker remains attached to the detectable label and/or nucleoside or nucleotide moiety after cleavage.

[0076] As used herein, “derivative” or “analog” means a synthetic nucleotide or nucleoside derivative having modified base moieties and/or modified sugar moieties. Such derivatives and analogs are discussed in, e.g., Scheit, *Nucleotide Analogs* (John Wiley & Son, 1980) and Uhlman *et al.*, *Chemical Reviews* 90:543-584, 1990. Nucleotide analogs can also comprise modified phosphodiester linkages, including phosphorothioate, phosphorodithioate, alkyl-phosphonate, phosphoranilidate and phosphoramidate linkages. “Derivative,” “analog” and “modified” as used herein, may be used interchangeably, and are encompassed by the terms “nucleotide” and “nucleoside” defined herein.

[0077] As used herein, the term “phosphate” is used in its ordinary sense as understood

by those skilled in the art, and includes its protonated forms (for example,  and ). As used herein, the terms “monophosphate,” “diphosphate,” and “triphosphate” are used in their ordinary sense as understood by those skilled in the art, and include protonated forms.

[0078] The terms “protecting group” and “protecting groups” as used herein refer to any atom or group of atoms that is added to a molecule in order to prevent existing groups in the molecule from undergoing unwanted chemical reactions. Sometimes, “protecting group” and “blocking group” can be used interchangeably.

[0079] As used herein, the term “phasing” refers to a phenomenon in SBS that is caused by incomplete removal of the 3' terminators and fluorophores, and failure to complete the

incorporation of a portion of DNA strands within clusters by polymerases at a given sequencing cycle. Pre-phasing is caused by the incorporation of nucleotides without effective 3' terminators, wherein the incorporation event goes 1 cycle ahead due to a termination failure. Phasing and pre-phasing cause the measured signal intensities for a specific cycle to consist of the signal from the current cycle as well as noise from the preceding and following cycles. As the number of cycles increases, the fraction of sequences per cluster affected by phasing and pre-phasing increases, hampering the identification of the correct base. Pre-phasing can be caused by the presence of a trace amount of unprotected or unblocked 3'-OH nucleotides during sequencing by synthesis (SBS). The unprotected 3'-OH nucleotides could be generated during the manufacturing processes or possibly during the storage and reagent handling processes. Accordingly, the discovery of nucleotide analogues which decrease the incidence of pre-phasing is surprising and provides a great advantage in SBS applications over existing nucleotide analogues. For example, the nucleotide analogues provided can result in faster SBS cycle time, lower phasing and pre-phasing values, and longer sequencing read lengths.

Sequencing Methods Utilizing Transition Metal Catalysts, Non-reducing Ligands, and Reducing Agents

[0080] Some embodiments of the present disclosure relate to a method for determining sequences of a plurality of target polynucleotides (e.g., single-stranded polynucleotides), comprising:

(a) contacting a solid support with sequencing primers under hybridization conditions, wherein the solid support comprises a plurality of target polynucleotides immobilized thereon; and the sequencing primers are complementary to at least a portion of the target polynucleotides;

(b) contacting the solid support with a first aqueous solution comprising DNA polymerase and one or more of four different types of nucleotides under conditions suitable for DNA polymerase-mediated primer extension, wherein each of the nucleotides comprises a 3' blocking group comprising an unsubstituted or substituted allyl group;

(c) incorporating one type of nucleotides into the sequencing primers to produce extended copy polynucleotides;

(d) performing one or more fluorescent measurements of the extended copy polynucleotides;

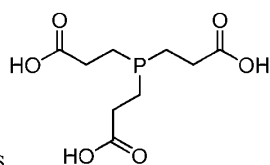
(e) removing the 3' blocking group of the incorporated nucleotides in a second aqueous solution comprising a transition metal catalyst and one or more reducing agents,

the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand; and

(f) washing the solid support with a third aqueous solution after the removal of the 3' blocking group of the incorporated nucleotides.

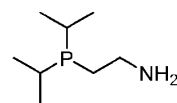
[0081] In some embodiments of the method described herein, the method further comprises repeating steps (b) through (f) until sequences of at least a portion of the target polynucleotides are determined. In a further embodiment, steps (b) through (f) are repeated at least 50 times, at least 100 times, at least 150 times, at least 200 times, at least 250 times, at least 300 times, at least 350 times, at least 400 times, at least 450 times or at least 500 times.

[0082] In some embodiments of the method described herein, the transition metal catalyst is a Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the water-soluble non-reducing phosphine. In further embodiments, the Pd(0) catalyst is generated *in situ* from the palladium complex (e.g., a Pd(II) complex) and a water soluble non-reducing phosphine. In some embodiments, the water soluble non-reducing phosphine comprises tris(substituted C₁-C₆ alkyl)phosphine or unsubstituted or substituted aromatic phosphine, or a combination thereof. In some embodiments, the transition metal catalyst is a Pd(0) catalyst generated *in situ* from the palladium complex and the water soluble non-reducing tris(substituted C₁-C₆ alkyl)phosphine. In other embodiments, the transition metal catalyst is a Pd(0) catalyst generated *in situ* from the palladium complex and the water soluble non-reducing tris(substituted C₆-C₁₀ aryl)phosphine. In some embodiments, the palladium complex comprises [Pd(Allyl)Cl]₂, Na₂PdCl₄, K₂PdCl₄, Li₂PdCl₄, [Pd(Allyl)(THPP)]Cl, [Pd(Allyl)(THPP)]₂Cl, Pd(CH₃CN)₂Cl₂, Pd(OAc)₂, Pd(PPh₃)₄, Pd(dba)₂, Pd(Acac)₂, PdCl₂(COD), Pd(TFA)₂, Na₂PdBr₄, K₂PdBr₄, PdCl₂, PdBr₂, or Pd(NO₃)₂, or combinations thereof. In further embodiments, the palladium complex comprises [Pd(Allyl)Cl]₂ or Na₂PdCl₄. In some embodiments, the tris(substituted C₁-C₆ alkyl)phosphine is a tris(carboxy substituted C₁-C₆ alkyl)phosphine, in which one, two or three C₁-C₆ alkyl can be substituted with a carboxy group. In one example, the tris(substituted C₁-C₆

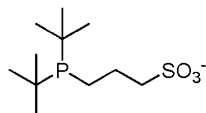


alkyl)phosphine is (TCEP). In some other embodiments, one, two or three C₁-C₆ alkyl of the tris(substituted C₁-C₆ alkyl)phosphine ligand may be substituted with one or more functional groups that improves the water solubility of the phosphine, such as amino, substituted amino, ammonium, -SO₃H, or sulfonate (-SO₃⁻), or combinations thereof. In some

embodiments, the tris(substituted C₁-C₆ alkyl)phosphine is:



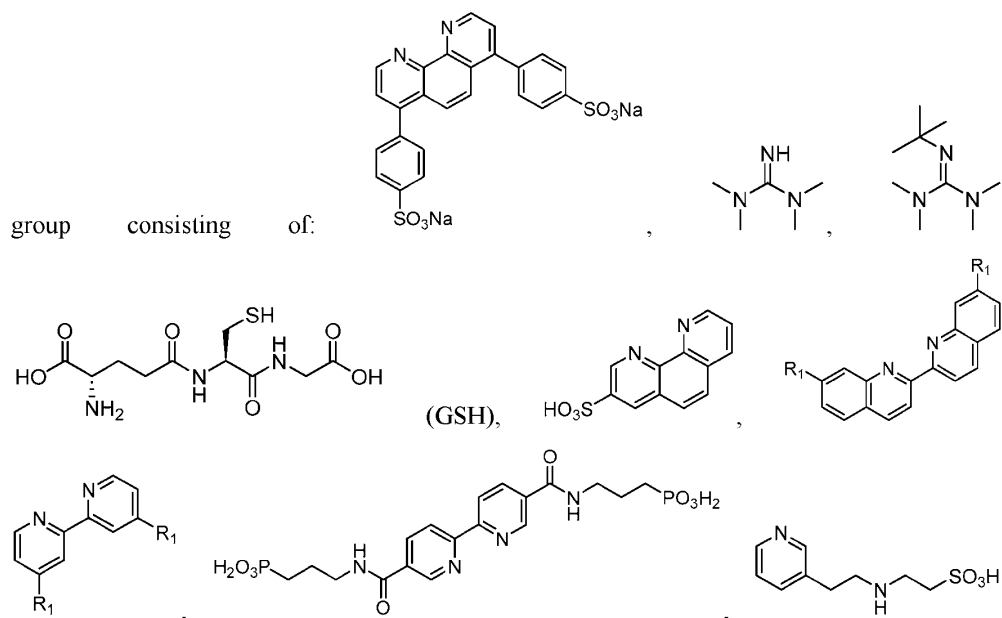
(DIPA) or



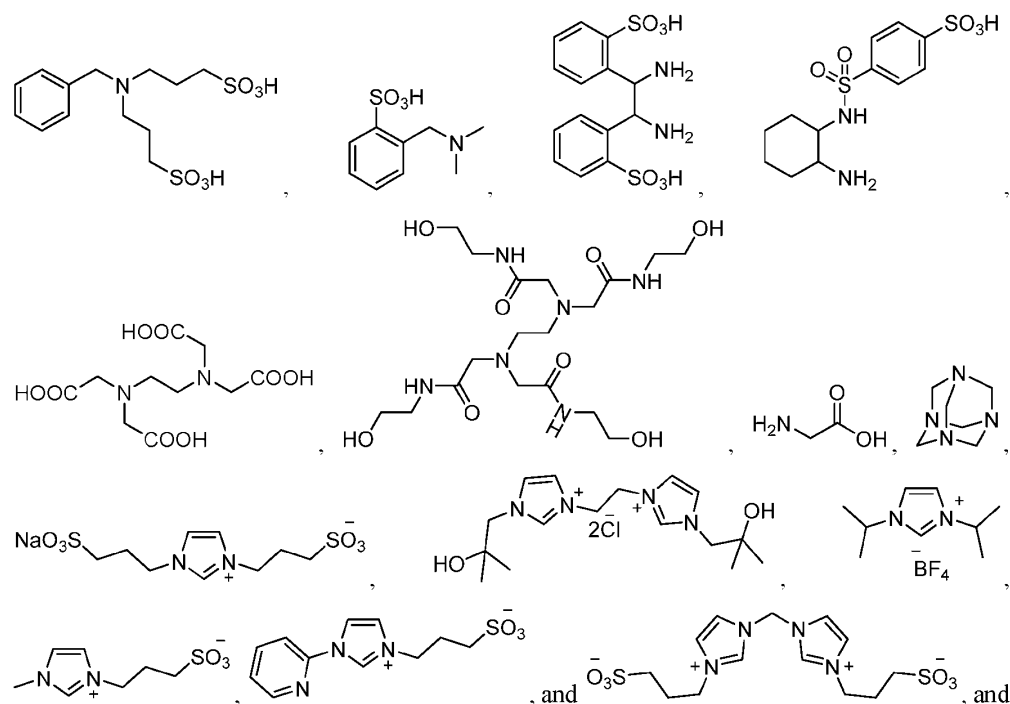
(diBuPpropSO₃⁻). In some embodiments, the non-reducing aromatic

phosphine comprises or is a tris(substituted C₆-C₁₀ aryl)phosphine, for example, a tris(substituted phenyl)phosphine, in which one, two or three phenyl groups can be substituted with one or more functional groups that improves the water solubility of the phosphine, such as amino, substituted amino, ammonium, -SO₃H, or sulfonate, or combinations thereof. In some embodiments, the tris(substituted phenyl)phosphine is tris(sulfonate substituted phenyl)phosphine. Such sulfonate substituted triphenyl phosphine may be in a salt form, such as a sodium or potassium salt. In addition, one, two or three phenyl groups of the tris(sulfonate substituted phenyl)phosphine may further be substituted with a carboxyl group. In some embodiments of the method, the molar ratio of the palladium complex and the water soluble phosphine is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10.

[0083] In some embodiments of the method, the transition metal catalyst is Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the N,N-bidentate non-phosphine ligand. In further embodiments, the N,N-bidentate ligand is selected from the

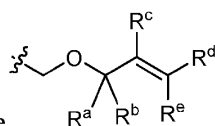




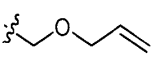


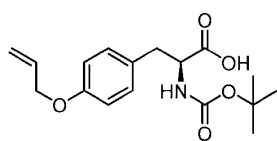
salts thereof; wherein R₁ is independently -SO₃H, -COOH, -PO₃H₂, or -NMe₃⁺; R₂ is -(CH₂)₁₋₃COOH; and R₃ is -SO₃H or -COOH. In some embodiments, the molar ratio of the palladium complex and the N,N-bidentate non-phosphine ligand is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10.

[0084] In some embodiments of the method, the reducing agent is a boron-containing reducing agent. In some embodiments, the boron-containing reducing agent is NH_3BH_3 or $\text{B}_2(\text{OH})_4$. In some embodiments, the boron-containing reducing agent is also phosphine reducing agent. Non limiting examples of the boron-containing phosphine reducing agent include sodium borohydride, borane tetrahydrofuran, lithium borohydride, sodium triacetoxymethylborohydride, borane dimethylamine, borane dimethyl sulfide, catecholborane, tetrabutylammonium borohydride, borane-ammonia complex, calcium borohydride, magnesium borohydride, potassium borohydride, dichlorophenylborane, calcium borohydride bis(tetrahydrofuran), potassium triethylborohydride, borane diphenylphosphine complex, dicyclohexylidoborane, tetraethylammonium borohydride, dichloro(diisopropylamino)borane, bromodimethylborane, diethylmethoxyborane, dichloromethyldiisopropoxyborane, bromodimethylborane, or monobromoborane methyl sulfide. In some embodiments of the method, the pH of the second aqueous solution is from about 7.0 to about 10, or from about 7.5 to about 9.5. In some embodiments of

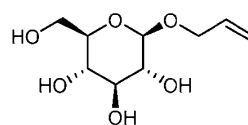


the method, the 3' blocking group has the structure attached to the 3' oxygen of the nucleotide, wherein each of R^a , R^b , R^c , R^d and R^e is independently H, halogen, unsubstituted or substituted C_1 - C_6 alkyl, or C_1 - C_6 haloalkyl. In further embodiments, the 3' blocking group of

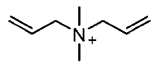
the nucleotide has the structure  attached to the 3' oxygen of the nucleotide. In some embodiments of the method, the first aqueous solution or the third aqueous solution comprises at least one Pd(0) scavenger. In yet a further embodiment, the Pd(0) scavenger comprises one or more allyl moieties selected from the group consisting of -O-allyl, -S-allyl, -NR-allyl, and -N⁺RR'-allyl, and combinations thereof, wherein R is H, unsubstituted or substituted C_1 - C_6 alkyl, unsubstituted or substituted C_2 - C_6 alkenyl, unsubstituted or substituted C_2 - C_6 alkynyl, unsubstituted or substituted C_6 - C_{10} aryl, unsubstituted or substituted 5 to 10 membered heteroaryl, unsubstituted or substituted C_3 - C_{10} carbocyclyl, or unsubstituted or substituted 5 to 10 membered heterocyclyl; and R' is H, unsubstituted C_1 - C_6 alkyl or substituted C_1 - C_6 alkyl. In yet a further embodiment, the Pd(0) scavenger comprising one or more -O-allyl moieties is



(N-Boc tyrosine(allyl)-OH) or



(allyl- β -D-glucopyranoside), or a salt thereof. In yet a further embodiment, the Pd(0) scavenger comprising

one or more one or more -NR-allyl or -N⁺RR'-allyl moieties is  Z^- , wherein Z^- is Cl^- or F^- . In a further embodiment, the Pd(0) scavenger comprising one or more allyl moieties is in the first aqueous solution. In some embodiments of the method, the third aqueous solution further comprises at least one Pd(II) scavenger. In a further embodiment, the Pd(II) scavenger comprises L-cysteine or sodium thiosulfate. In some embodiments of the method, the 3' blocking group of the incorporated nucleotides is removed in less than 5 minutes when step (e) is performed at room temperature. In some embodiments of the method, the 3' blocking group of the incorporated nucleotides is removed in about 1 minute. In some embodiments of the method, the solid support comprises an array of immobilized target polynucleotides.

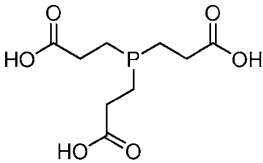
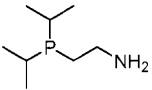
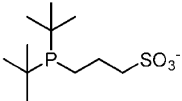
Non-Reducing Ligands

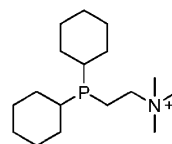
[0085] In some embodiments, ligands included to generate a transition metal catalyst are non-reducing ligands. In some embodiments, non-reducing ligands may include water soluble non-reducing phosphines. In some other embodiments, non-reducing ligands may include a N,N-

bidentate non-phosphine ligand. A transition metal complex (pre-catalyst) may be mixed with a non-reducing ligand to create a coordination complex. Separately, at least one reducing agent is required to reduce the transition metal phosphine ligand coordination complex into the active catalyst form. For example, a Pd(II) pre-catalyst $[\text{Pd}(\text{Allyl})\text{Cl}]_2$ or Na_2PdCl_4 can form an intermediate Pd(II) coordination complex with a non-reducing phosphine TCEP. In order for the Pd(II) intermediate complex to be transformed into the active catalytic form, a reducing agent (such as a boron-containing reducing agent described herein) is also introduced, reducing Pd(II) into the active Pd(0) form. Under certain reaction conditions, TCEP by itself may not serve as a reducing agent due to it does not have the correct potential to reduce a Pd(II) complex. Such a reaction pathway may be desirable, as it decouples reduction of the transition metal from coordination of the transition metal. In contrast, certain phosphine ligand may serve the dual purpose of coordinating with the transition metal and reducing the transition metal into the active catalytic form. For example, when Na_2PdCl_4 is mixed with tris(hydroxypropyl)phosphine (THPP or THP), THPP also serves as a reducing agent to reduce Pd(II) into Pd(0).

[0086] Non-reducing water-soluble phosphine ligands, include, for example tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphines, where one, two or one, two or three $\text{C}_1\text{-C}_6$ alkyl of the tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphine ligand may be substituted with one or more functional groups that improves the water solubility of the phosphine, such as amino, substituted amino, ammonium,

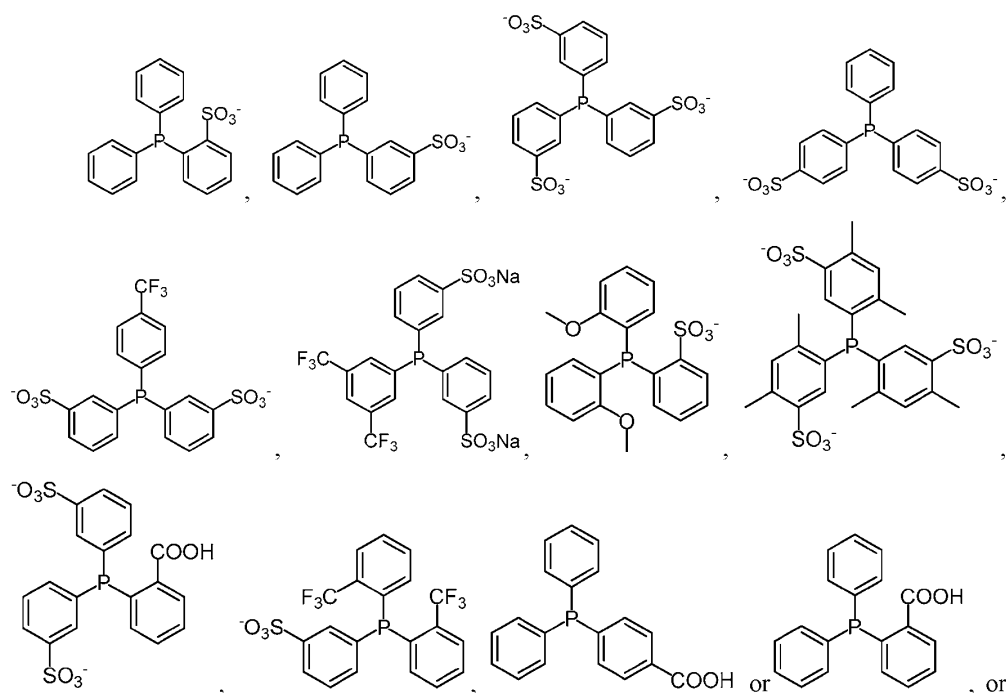
$-\text{SO}_3\text{H}$, or sulfonate, or combinations thereof; while these functional groups cannot serve as electron donor to the transition metal, for example, to reduce Pd(II) into Pd(0). Such tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphines may include at least one substituted $\text{C}_1\text{-C}_6$ alkyl, but all three $\text{C}_1\text{-C}_6$ alkyl need not be substituted. Suitable tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphines may

include tris(carboxyethyl)phosphine  (TCEP), 2-(diisopropylphosphanyl)ethan-1-amine  (DIPA), and 3-(di-*tert*butylphosphanyl)propane-1-sulfonate  (diBuPpropSO₃⁻). Other suitable non-reducing phosphine may be a (substituted $\text{C}_1\text{-C}_6$ alkyl)(dicycloalkyl)phosphine, such as 2-

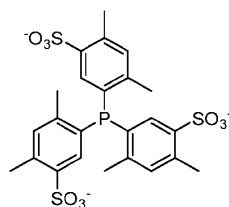
(dicyclohexylphosphanyl)-*N,N,N*-trimethylethan-1-aminium

(DCPT).

Additional suitable non-reducing phosphine may be aromatic phosphines, such as tris(substituted C₆-C₁₀ aryl)phosphine or tris(substituted phenyl)phosphine, in which one, two or three phenyl groups can be substituted with one or more functional groups that improves the water solubility of the phosphine, such as amino, substituted amino, ammonium, -SO₃H, or sulfonate, or combinations thereof. In some embodiments, the tris(substituted phenyl)phosphine is tris(sulfonate substituted phenyl)phosphine. Such sulfonate substituted triphenyl phosphine may be in a salt form, such as a sodium or potassium salt. In addition, one, two or three phenyl groups of the tris(sulfonate substituted phenyl)phosphine may further be substituted with a carboxyl group. Exemplary tris(substituted phenyl)phosphine includes but not limited to:



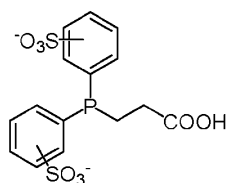
salts thereof, such as sodium or potassium salt, for example, tris(2,4-dimethyl-5-



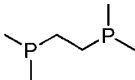
sulfophenyl)phosphine trisodium salt

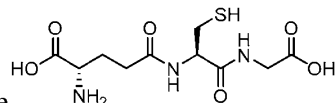
(TXPTS). Additional suitable non-

reducing phosphine may be (substituted diphenyl)(substituted C₁-C₆ alkyl)phosphine, such as

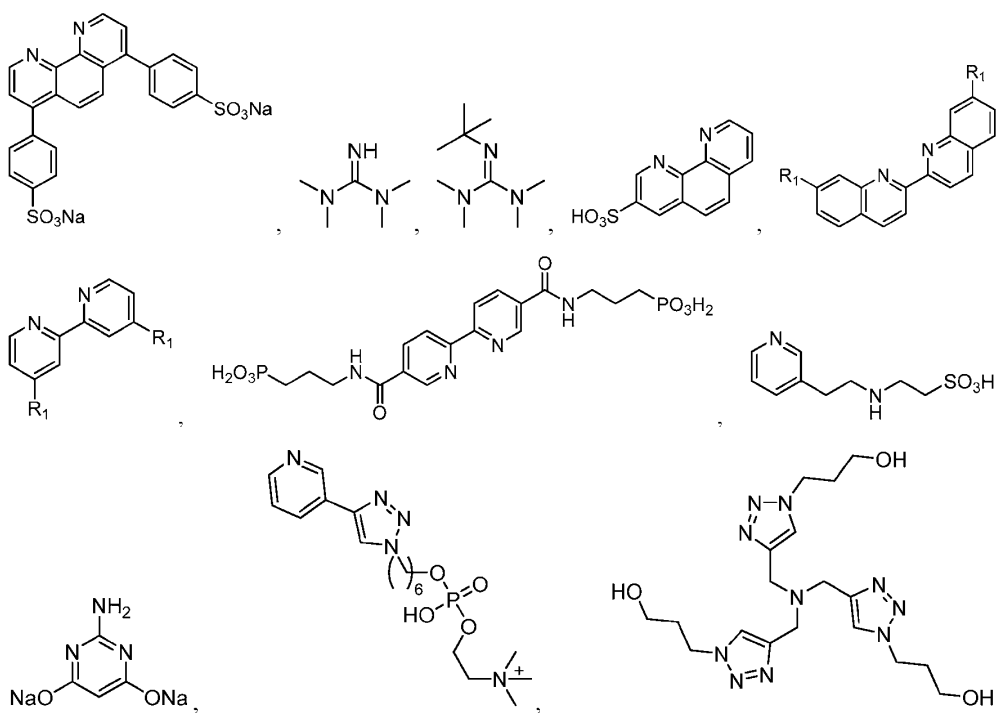


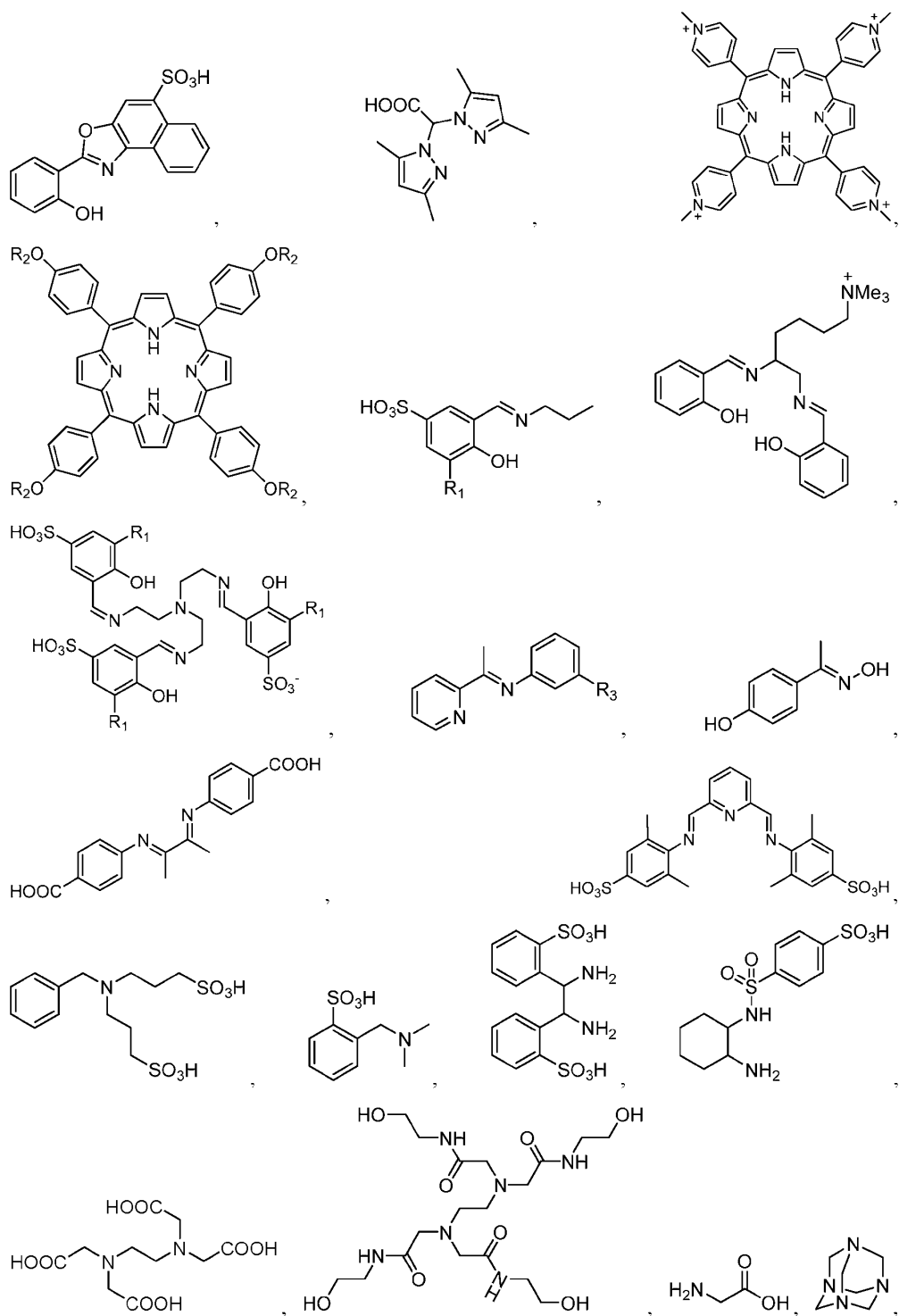
. Other water soluble phosphines may be suitable non-reducing agents. For

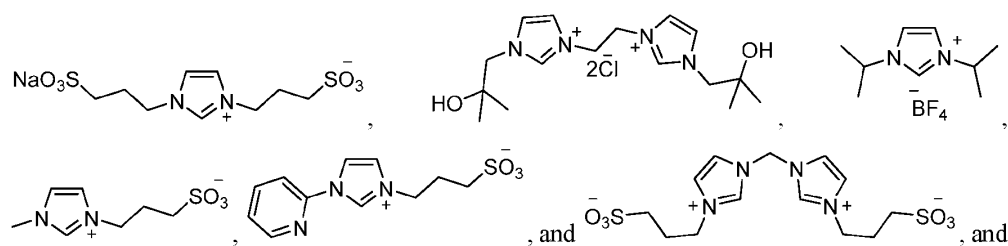
example, 1,2-bis(dimethylphosphanyl)ethane  ((diMeP)₂Et) may be a suitable non-reducing ligand. Some non-phosphine molecules may be suitable non-reducing ligands, such as

N,N-bidentate ligands. In some embodiments, glutathione  (GSH) may be a suitable non-reducing ligand.

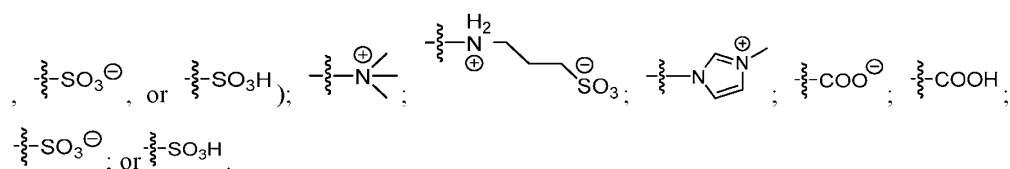
[0087] Other suitable N,N-bidentate ligand may include, for example:







salts thereof, wherein R₁ is independently -SO₃H, -COOH, -PO₃H₂, or -NMe₃⁺; R₂ is -(CH₂)₁₋₃COOH; and R₃ is -SO₃H or -COOH. In some other embodiments, the N,N-bidentate ligands described herein may contain additional water soluble group(s), including but not limited to phosphate, $\text{--}\overset{\oplus}{\text{N}}\text{H}_3$, $\text{--}\overset{\oplus}{\text{N}}(\text{C}_1\text{--C}_6 \text{ alkyl})_3$ (wherein each C₁–C₆ alkyl in $\text{--}\overset{\oplus}{\text{N}}(\text{C}_1\text{--C}_6 \text{ alkyl})_3$ is optionally substituted with one or more substituents selected from phosphate, $\text{--COO}^\ominus$, --COOH



Reducing Agents

[0088] A reducing agent may be used to reduce a transition metal from its pre-catalytic form to the active form for a 3' deblocking reaction and/or cleavage of the linker in accordance with the present disclosure. In some embodiments, the reducing agent contains boron. For example, the reducing agent may be a borane, a borinic acid, a boronic acid, a boronic ester, or a borohydride. In some embodiments, the reducing agent is a borane or a boronic acid. Suitable boranes include diborane, triborane, tetraborane, pentaborane, hexaborane, heptaborane, octaborane, nonaborane, decaborane, borane tetrahydrofuran, borane dimethyl sulfide, catecholborane, dichlorophenylborane, borane diphenylphosphine complex, dicyclohexyliodoborane, bromodimethylborane, diethylmethoxyborane, dichloromethyldiisopropoxyborane, bromodimethylborane, and mono-bromoborane methyl sulfide. Suitable boranes include amino-boranes. Suitable amine-boranes include pyridine-boranes, N-based heteroaromatic-borane complexes, aminetrihydridoboron (NH_3BH_3), borane-ammonia complex, borane dimethylamine, borane tert-butylamine, borane trimethylamine, borane isopropylamine, dichloro(diisopropylamino)borane, and borazine. Suitable boronic acids include boric acid, 4-hydroxyphenylboronic acid, tetrahydroxidoboron ($\text{B}_2(\text{OH})_4$), phenylboronic acid, 2-thienylboronic acid, methylboronic acid, *cis*-propenylboronic acid, and *trans*-propenylboronic acid. Suitable boronic esters include allylboronic acid pinacol ester, phenyl

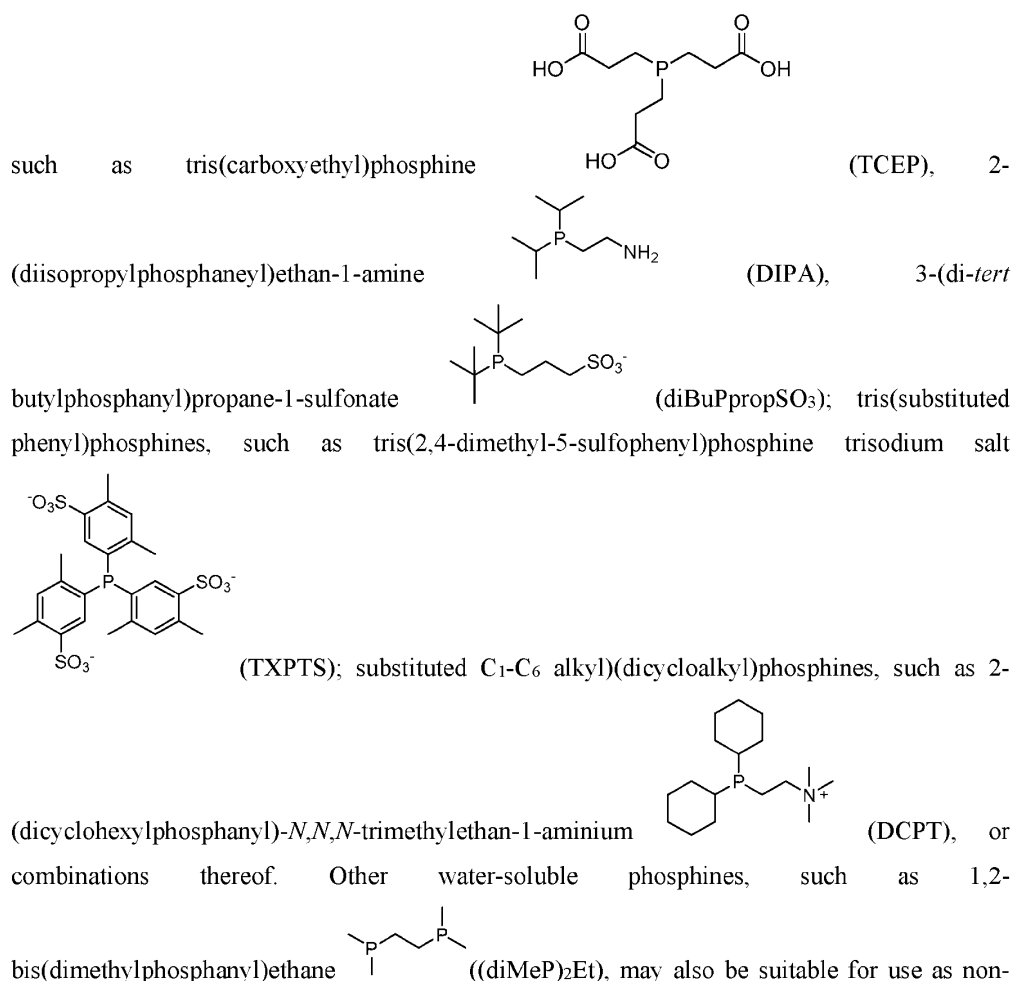
boronic acid trimethylene glycol ester, and diisopropoxymethylborane. Suitable borohydrides include sodium borohydride (Na_2BH_4), lithium borohydride, calcium borohydride, calcium borohydride bis(tetrahydrofuran), magnesium borohydride, potassium borohydride, potassium triethylborohydride, sodium triacetoxyborohydride, tetraethylammonium borohydride, and tetrabutylammonium borohydride. In some embodiments, the concentration of the reducing agent (such as boron-containing reducing agent) in the first aqueous solution (also called cleavage mixture) is about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM, 4.5 mM, 5 mM, 5.5 mM, 6 mM, 6.5 mM, 7 mM, 7.5 mM, 8 mM, 8.5 mM, 9 mM, 9.5 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM, 20 mM, 22.5 mM, 25 mM, 37.5 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM, 65 mM, 70 mM, or 75 mM, or a range defined by any two of the preceding values. In one embodiment, the concentration of the reducing agent in the first aqueous solution is about 20 mM.

Transition Metal Catalysts

[0089] Transition metal catalysts may be used in a reaction to remove a 3' blocking group, in conjunction with a non-reducing ligand and a reducing agent. Suitable transition metals for use as catalysts include palladium (Pd) and nickel (Ni). Similar to palladium, nickel precatalyst may exist in the form of Ni(II), and the active catalytic form is Ni(0).

Palladium Catalysts

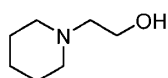
[0090] In some embodiments, the Pd catalyst used for removing or cleaving the 3' blocking group described herein is water soluble. In some such embodiments, the Pd catalyst is the active Pd(0) form. In some instances, the Pd(0) catalyst may be generated *in situ* from reduction of a Pd complex or Pd precatalyst (e.g., a Pd(II) complex) by reagents such as alkenes, alcohols, amines, phosphines, or metal hydrides. Suitable Pd sources include $\text{Pd}(\text{CH}_3\text{CN})_2\text{Cl}_2$, $[\text{Pd}(\text{Allyl})\text{Cl}]_2$, $[\text{Pd}(\text{Allyl})(\text{THPP})]\text{Cl}$, $[\text{Pd}(\text{Allyl})(\text{THPP})_2]\text{Cl}$, Na_2PdCl_4 , K_2PdCl_4 , Li_2PdCl_4 , $\text{Pd}(\text{OAc})_2$, $\text{Pd}(\text{PPh}_3)_4$, $\text{Pd}(\text{dba})_2$, $\text{Pd}(\text{Acac})_2$, $\text{PdCl}_2(\text{COD})$, $\text{Pd}(\text{TFA})_2$, Na_2PdBr_4 , K_2PdBr_4 , PdCl_2 , PdBr_2 , and $\text{Pd}(\text{NO}_3)_2$. In one such embodiment, the Pd(0) complex is generated *in situ* from an organic or inorganic salt of palladate (II), for example, Na_2PdCl_4 or K_2PdCl_4 . In another embodiment, the palladium source is allyl Pd(II) chloride dimer $[(\text{Allyl})\text{PdCl}]_2$ or $[\text{PdCl}(\text{C}_3\text{H}_5)]_2$. In some embodiments, the Pd(0) catalyst is generated in an aqueous solution by mixing a Pd(II) complex with a water soluble, non-reducing ligand and a reducing agent in accordance with the present disclosure. Suitable non-reducing ligands include tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphines,



[0091] In some embodiments, the palladium catalyst is prepared by mixing [(Allyl)PdCl]₂ with TCEP *in situ*. The molar ratio of [(Allyl)PdCl]₂ and the TCEP may be about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10. In one embodiment, the molar ratio of [(Allyl)PdCl]₂ to TCEP is 1:5. In some other embodiment, the palladium catalyst is prepared by mixing a water-soluble Pd reagent such as Na₂PdCl₄ or K₂PdCl₄ with TCEP *in situ*. The molar ratio of Na₂PdCl₄ or K₂PdCl₄ and TCEP may be about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10. In one embodiment, the molar ratio of Na₂PdCl₄ or K₂PdCl₄ to TCEP is about 1:3. In another embodiment, the molar ratio of Na₂PdCl₄ or K₂PdCl₄ to TCEP is about 1:3.5.

[0092] The Pd complex and the water-soluble phosphine for use in the cleavage step of the method described herein may be in a composition or a mixture, also called cleavage mix.

In some further embodiments, the cleavage mix may contain additional buffer reagents, such as a primary amine, a secondary amine, a tertiary amine, a natural amino acid, a non-natural amino acid, a carbonate salt, a phosphate salt, or a borate salt, or combinations thereof. In some further embodiments, the buffer reagent comprises ethanolamine (EA), tris(hydroxymethyl)aminomethane (Tris), glycine, sodium carbonate, sodium phosphate, sodium borate, dimethylethanolamine (DMEA), diethylethanolamine (DEEA), N,N,N',N'-tetramethylethylenediamine (TMEDA), N,N,N',N'-tetraethylethylenediamine (TEEDA), or piperidyl ethanolamine (PipEA or 1-(2-hydroxyethyl)piperidine, having the structure



), or combinations thereof. In one embodiment, the one or more buffer reagents comprise DEEA. In another embodiment, the one or more buffer reagents comprise PipEA. In another embodiment, the one or more buffer reagents contains one or more inorganic salts such as a carbonate salt, a phosphate salt, or a borate salt, or combinations thereof. In one embodiment, the inorganic salt is a sodium salt. In some embodiments, the molar ratio of the Pd catalyst to the reducing agent is about 1:100, 1:50, 1:20, 1:10 or 1:5. In some embodiments, the molar ratio of the Pd catalyst to the Pd scavenger comprising one or more allyl moieties is about 1:100, 1:50, 1:20, 1:10 or 1:5. In some further embodiments, the Pd scavenger comprises one or more allyl moieties is a Pd scavenger for Pd(0), the active form of the Pd catalyst.

[0093] In some embodiments, the cleavage condition for the 3' blocking group is the same as the condition for cleaving the cleavable linker of the nucleotide. For example, the nucleotide may comprise a linker moiety that is the same as the 3' blocking group. In other embodiments, the cleavage condition for the 3' blocking group is different from the condition for cleaving the cleavable linker of the nucleotide.

Nickel Catalysts

[0094] In some embodiments, the Ni catalyst used for removing or cleaving the 3' blocking group described herein is water soluble. In some such embodiments, the Ni complex is a Ni(0) complex. In some instances, the Ni(0) complex may be generated *in situ* from reduction of a Ni complex or Ni precatalyst (e.g., a Ni(II) complex) by reagents such as alkenes, alcohols, amines, phosphines, or metal hydrides. In some embodiments, the Ni(II) compound is NiCl₂. In some embodiments, the Ni(0) catalyst is generated in an aqueous solution by mixing a Ni(II) complex with a water soluble, non-reducing ligand and a reducing agent in accordance with the present disclosure. For example, the Ni complex is prepared by mixing NiCl₂ with 1 to 10 molar equivalents of a water-soluble non-reducing ligand. Suitable non-reducing ligands include tris(substituted C₁-C₆ alkyl)phosphines, such as TCEP, DIPA, diBuPpropSO₃, TXPTS, DCPT, or combinations thereof. Other water-soluble phosphines, such as 1,2-

bis(dimethylphosphanyl)ethane ((diMeP)₂Et), may also be suitable for use as non-reducing ligands. Suitable reducing agents may contain borane, in accordance with the present disclosure. In some embodiments, the Ni(0) catalyst is generated in an aqueous solution by mixing a Ni(II) complex with a water soluble, reducing ligand (e.g., THPP) in accordance with the present disclosure.

Palladium Scavengers

[0095] Certain aspects of the present disclosure relate to employing alternative palladium scavengers in several steps of sequencing by synthesis, where at least one palladium scavenger comprises one or more allyl moieties (e.g., -O-allyl, -S-allyl, -NR-allyl, or -N⁺RR'-allyl), or combinations thereof, acting as a competitive substrate to consume any residual Pd(0) sticking on the nucleic acid (i.e., a Pd(0) scavenger). These palladium scavengers are described in WO/2022/243480, which is incorporated by reference in its entirety. The sequencing methods described herein substantially improve the sequencing metrics (e.g., reduce phasing and prephasing values) and may also reduce the sequencing time for each cycle by certain eliminating post-cleavage treatment step.

[0096] In some embodiments of any of the methods described herein, the palladium scavenger comprises one or more allyl moieties is in the first aqueous solution. In some instances, the first aqueous solution is also known as the incorporation mix (IMX). In some such embodiments, such palladium scavenger is compatible with the other sequencing reagents in the first aqueous solution, which may also include a polymerase (such as DNA polymerase), in addition to the one or more different types of nucleotides. In some such embodiments, the polymerase is a DNA polymerase, such as a mutant of 9°N polymerase (e.g., those disclosed in WO 2005/024010, which is incorporated by reference), for example, Pol 812, Pol 1901, Pol 1558 or Pol 963. The amino acid sequences of Pol 812, Pol 1901, Pol 1558 or Pol 963 DNA polymerases are described, for example, in U.S. Patent Publication Nos. 2020/0131484 A1 and 2020/0181587 A1, both of which are incorporated by reference herein. In some embodiments, the first aqueous solution further comprises one or more buffering agents. The buffering agents may comprise a primary amine, a secondary amine, a tertiary amine, a natural amino acid, or a non-natural amino acid, or combinations thereof. In further embodiments, the buffering agents comprise ethanolamine or glycine, or a combination thereof. In one embodiment, the buffer agent comprises or is glycine. In further embodiments, the palladium scavenger comprises one or more allyl moieties does not require a separate washing step prior to the next incorporation cycle. In further embodiments, the palladium scavenger in the first aqueous solution is a Pd(0) scavenger described herein. In some embodiments, the Pd(0) scavenger is premixed with the DNA polymerase and/or

the one or more of four types of nucleotides (e.g., dATP, dCTP, dGTP, and dTTP or dUTP). In other embodiments, the Pd(0) scavenger is stored separately from the DNA polymerase and/or the one or more of four types of nucleotides and is mixed with these components shortly before sequencing run starts.

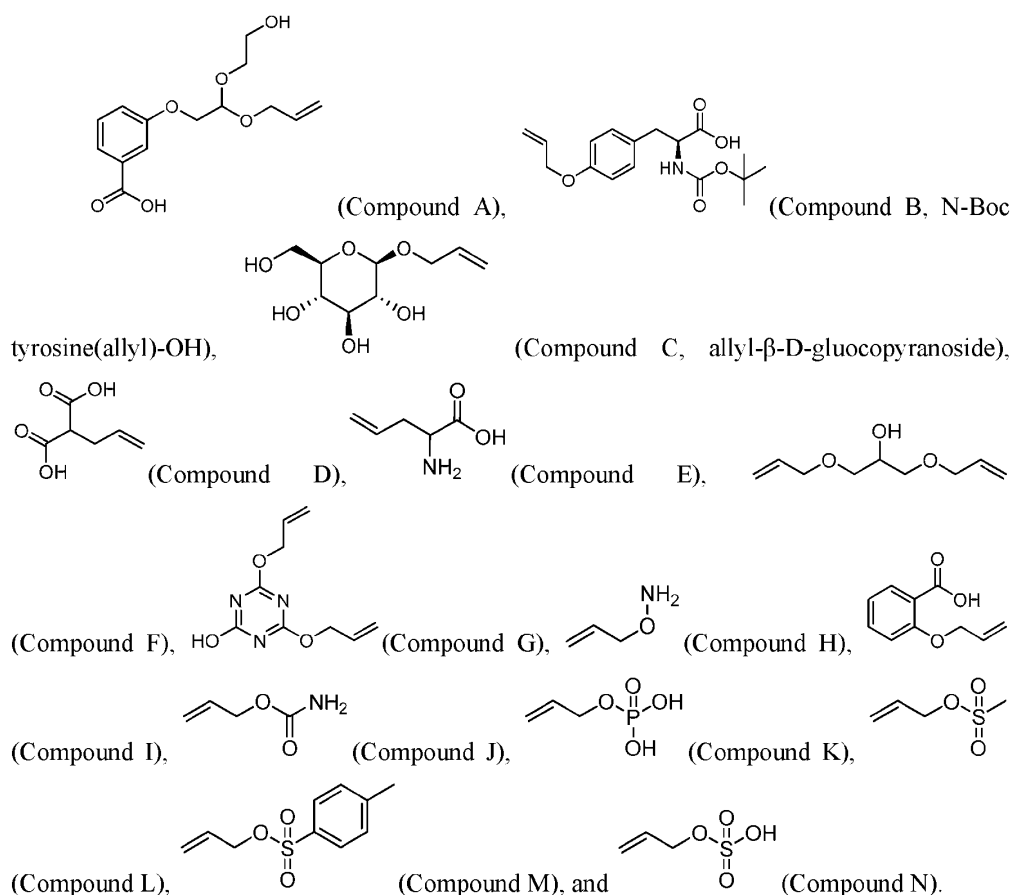
[0097] In some embodiments of any of the methods described herein, the concentration of the Pd(0) scavenger comprising one or more allyl moieties in the first aqueous solution is from about 0.1 mM to about 100 mM, from 0.2 mM to about 75 mM, from about 0.5 mM to about 50 mM, from about 1 mM to about 20 mM, or from about 2 mM to about 10 mM. In further embodiments, the concentration of the palladium scavenger (e.g., the Pd(0) scavenger) is about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM, 4.5 mM, 5 mM, 5.5 mM, 6 mM, 6.5 mM, 7 mM, 7.5 mM, 8 mM, 8.5 mM, 9 mM, 9.5 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM or 20 mM, or a range defined by any two of the preceding values. In further embodiments, the concentration of such palladium scavenger is the concentration in the first aqueous solution. In further embodiments, the pH of the first aqueous solution is about 9.

[0098] In some other embodiments of any of the methods described herein, the palladium scavenger comprises one or more allyl moieties in a solution when performing one or more fluorescent measurements. In such embodiment, such palladium scavenger is compatible with the sequencing reagents of the scanning solution (also known as the scan mix). In further embodiments, the one or more palladium scavengers does not require a separate washing step prior to the next incorporation cycle. In further embodiments, the palladium scavenger in the scan solution is a Pd(0) scavenger described herein.

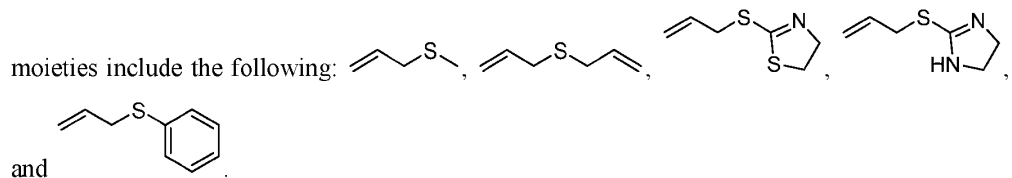
[0099] In other embodiments of the methods described herein, the palladium scavenger comprises one or more allyl moieties in the post cleavage wash solution (i.e., the second aqueous solution). In further embodiments, the palladium scavenger in the post cleavage wash solution is a Pd(0) scavenger described herein. In some such embodiment, the post cleavage wash solution does not comprise lipoic acid or 3,3'-dithiodipropionic acid (DDPA).

[0100] In still other embodiments of the method described herein, the palladium scavenger comprises one or more allyl moieties may be present both in the first aqueous solution (e.g., incorporation mix) and in the second aqueous solution (e.g., post cleavage wash solution), or present in both the first aqueous solution and the scan mix. In some such embodiment, the post cleavage wash solution does not comprise lipoic acid or DDPA.

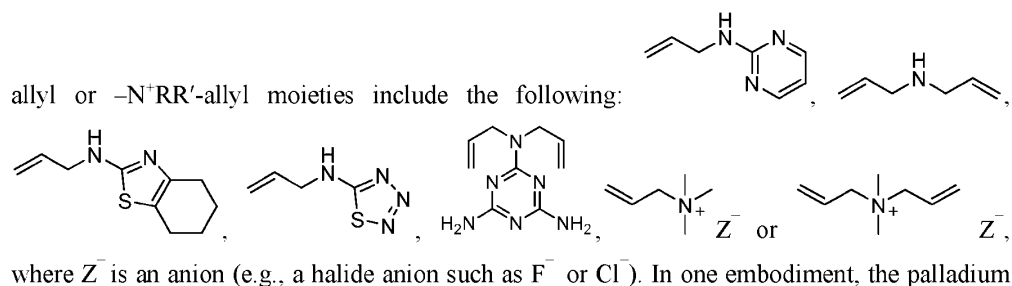
[0101] Non-limiting examples of the Pd(0) scavenger comprising one or more -O-allyl or allyl moieties include the following:

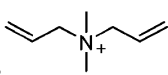


[0102] Non-limiting examples of the Pd(0) scavenger comprising one or more –S-allyl



[0103] Non-limiting examples of the Pd(0) scavenger comprising one or more –NR-



scavenger is  Cl⁻ (Compound O, diallyldimethylammonium chloride, also known as DADMAC).

[0104] In some embodiments of the methods described herein, the method may further use additional palladium scavenger(s), such as Pd(II) scavenger(s). In some such embodiments, the use of additional Pd scavenger(s) may improve the phasing value of the sequencing metrics. For example, the additional Pd scavenger(s) may comprise an isocyanoacetate (ICNA) salt, ethyl isocyanoacetate, methyl isocyanoacetate, cysteine (e.g., L-cysteine) or a salt thereof (e.g., *N*-acetyl-L-cysteine), potassium ethylxanthogenate, potassium isopropyl xanthate, glutathione, ethylenediaminetetraacetic acid (EDTA), iminodiacetic acid, nitrilodiacetic acid, trimercapto-S-triazine, dimethyldithiocarbamate, dithiothreitol, mercaptoethanol, allyl alcohol, propargyl alcohol, thiol, thiosulfate salt (e.g., sodium thiosulfate or potassium thiosulfate), tertiary amine and/or tertiary phosphine, or combinations thereof. In one embodiment, the method also includes the use of L-cysteine or a salt thereof. In another embodiment, the method also includes the use of a thiosulfate salt such as sodium thiosulfate (Na₂S₂O₃). In some embodiments, the additional Pd scavenger is a scavenger for Pd(II). In some such embodiments, the Pd(II) scavenger (e.g., L-cysteine or sodium thiosulfate) is in the first aqueous solution. In other embodiments, the Pd(II) scavenger (e.g., L-cysteine or sodium thiosulfate) is in the post cleavage wash solution (i.e., the second aqueous solution). In other embodiments, the Pd(II) scavenger (e.g., L-cysteine or sodium thiosulfate) may be present both in the first aqueous solution and the second aqueous solution. In other embodiments, the Pd(II) scavenger (e.g., L-cysteine or sodium thiosulfate) may be present in the scan mixture (i.e., the solution in which one or more fluorescent measurements of the incorporated nucleotide are performed). In other embodiments, the Pd(II) scavenger may be present in one or more of incorporation mixture (e.g., the first aqueous solution), the scan mixture, or the post-cleavage wash solution (e.g., the second aqueous solution). In further embodiments, the concentration of the Pd(II) scavenger such as L-cysteine or sodium thiosulfate in the first aqueous solution or the second aqueous solution is from about 0.1 mM to about 100 mM, from 0.2 mM to about 75 mM, from about 0.5 mM to about 50 mM, from about 1 mM to about 20 mM, or from about 2 mM to about 10 mM. In further embodiments, the concentration of the Pd(II) scavenger such as L-cysteine or sodium thiosulfate is about 0.1 mM, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 6.5 mM, 7 mM, 8 mM, 9 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM or 20 mM, or a range defined by any two of the preceding values. In further embodiments, the Pd(II) scavenger is in the second aqueous solution, and the concentration of the Pd(II) scavenger in the second aqueous solution is about 10 mM.

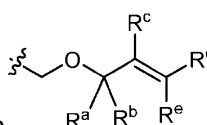
[0105] In some embodiments of the methods described herein, all Pd scavengers are in the first aqueous solution. In some other embodiments of the methods described herein, all Pd scavengers are in the second aqueous solution. In some other embodiments, the one or more Pd scavenger comprising one or more allyl moieties (e.g., Pd(0) scavenger) is in the incorporation mixture (i.e., first aqueous solution), and the Pd(II) scavenger(s) is in the post cleavage wash solution (i.e., second aqueous solution). In further embodiment, the post cleavage wash solution does not contain lipoic acid or DDPA. In other embodiments, the method does not include a post-cleavage wash step.

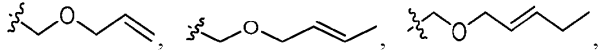
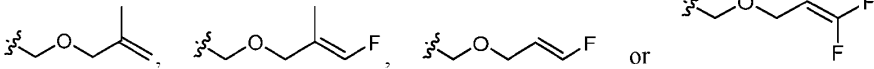
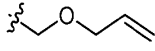
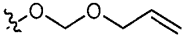
[0106] In some embodiments of the methods described herein, the target polynucleotide is immobilized to a surface of a substrate. In some further embodiments, the surface comprises a plurality of immobilized target polynucleotides, for example, an array of different immobilized target polynucleotides. In some such embodiments, the substrate comprises glass, modified or functionalized glass, plastics, polysaccharides, nylon, nitrocellulose, resins, silica, silicon, modified silicon, carbon, metals, inorganic glasses, or optical fiber bundles, or combinations thereof. In some further embodiments, the substrate is a flowcell, a nanoparticle, or a bead (such as spherical silica beads, inorganic nanoparticles, magnetic nanoparticles, cadmium-based dots, and cadmium free dots, or a bead disclosed in U.S. Publication No. 2021/0187470 A1, which is incorporated by reference). In one embodiment, the substrate is a flowcell comprising patterned nanowells separated by interstitial regions, and wherein the immobilized target polynucleotides reside inside the patterned nanowells.

[0107] In some embodiments of any of the methods described herein, the method is performed on an automated sequencing instrument, and wherein the automated sequencing instrument comprises two light sources operating at different wavelengths (e.g., at about 450 nm to about 460 nm, and about 520 nm to about 540 nm, in particular at about 460 nm and about 532 nm). In other embodiments, the automated sequencing instrument comprises a single light source operating at one wavelength.

Nucleotides with 3' Blocking Groups

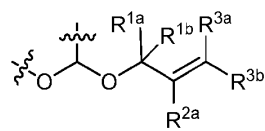
[0108] Some embodiments of the present disclosure relate to a nucleotide molecule comprising a nucleobase, a ribose or deoxyribose moiety, and a 3' hydroxy blocking group comprising an unsubstituted or substituted allyl moiety, such as a 3' blocking group having the

structure  attached to the 3' oxygen of the nucleotide, wherein each of R^a, R^b, R^c, R^d and R^e is independently H, halogen, unsubstituted or substituted C₁-C₆ alkyl, or C₁-C₆

haloalkyl. In one embodiment, each of R^a , R^b , R^c , R^d and R^e is H. In some other embodiments, each of R^a and R^b is H and at least one of R^c , R^d and R^e is independently halogen (e.g., fluoro, chloro) or unsubstituted C_1 - C_6 alkyl (e.g., methyl, ethyl, isopropyl, isobutyl, or t-butyl). For example, R^c is unsubstituted C_1 - C_6 alkyl and each of R^d and R^e is H. In another example, R^c is H and one or both of R^d and R^e is halogen or unsubstituted C_1 - C_6 alkyl. Non-limiting embodiments of the 3' blocking group include , . In one embodiment, the 3' blocking group is , and together with the 3' oxygen it forms  ("AOM") group attached to the 3' carbon atom of the ribose or deoxyribose moiety. Additional embodiments of the 3' blocking groups are described in U.S. Publication No. 2020/0216891 A1, which is incorporated by reference in its entirety. In any embodiments of the nucleotide described herein, the nucleotide may comprise a 3' blocked 2-deoxyribose moiety. Furthermore, the nucleotide may be a nucleoside triphosphate. In another embodiment, the 3' blocking group is an allyl ether group ($-O-CH_2CH=CH_2$), attached to the 3' carbon atom of the deoxyribose moiety.

Labeled Nucleotides

[0109] In some embodiments, the 3' blocked nucleotide also comprises a detectable label and such nucleotide is called a labeled nucleotide or a fully functionalized nucleotide (ffN). The label (e.g., a fluorescent dye) is conjugated via a cleavable linker by a variety of means including hydrophobic attraction, ionic attraction, and covalent attachment. In some aspect, the dyes are conjugated to the nucleotide by covalent attachment via the cleavable linker. One of ordinary skill in the art understands that label may be covalently bounded to the linker by reacting a functional group of the label (e.g., carboxyl) with a functional group of the linker (e.g., amino). In some such embodiments, the cleavable linker may comprise a moiety that is the same as the 3' blocking group. As such, the cleavable linker and the 3' blocking group may be cleaved or removed under the same reaction condition. In some such embodiments, the cleavable linker may comprise an allyl moiety, more particularly comprises a moiety of the structure:



, wherein each of R^{1a} , R^{1b} , R^{2a} , R^{3a} and R^{3b} is independently H, halogen, unsubstituted or substituted C_1 - C_6 alkyl, or C_1 - C_6 haloalkyl.

[0110] In some embodiments, the dye may be covalently attached to oligonucleotides or nucleotides via the nucleotide base. For example, the labeled nucleotide or oligonucleotide may have the label attached to the C5 position of a pyrimidine base or the C7 position of a 7-deaza purine base through a cleavable linker moiety.

[0111] Nucleotides may be labeled at sites on the sugar or nucleobase. As known in the art, a “nucleotide” consists of a nitrogenous base, a sugar, and one or more phosphate groups. In RNA, the sugar is ribose and in DNA is a deoxyribose, i.e., a sugar lacking a hydroxy group that is present in ribose. The nitrogenous base is a derivative of purine (e.g., deazapurine, 7-deazapurine) or pyrimidine. The purines are adenine (A) and guanine (G), and the pyrimidines are cytosine (C) and thymine (T) or in the context of RNA, uracil (U). The C-1 atom of deoxyribose is bonded to N-1 of a pyrimidine or N-9 of a purine. A nucleotide is also a phosphate ester of a nucleoside, with esterification occurring on the hydroxy group attached to the C-3 or C-5 of the sugar. Nucleotides are usually mono, di- or triphosphates.

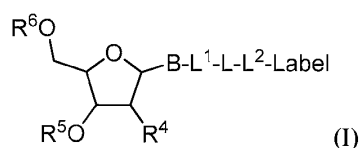
[0112] Although the base is usually referred to as a purine or pyrimidine, the skilled person will appreciate that derivatives and analogues are available which do not alter the capability of the nucleotide or nucleoside to undergo Watson-Crick base pairing. “Derivative” or “analogue” means a compound or molecule whose core structure is the same as, or closely resembles that of a parent compound but which has a chemical or physical modification, such as, for example, a different or additional side group, which allows the derivative nucleotide or nucleoside to be linked to another molecule. For example, the base may be a deazapurine. In particular embodiments, the derivatives should be capable of undergoing Watson-Crick pairing. “Derivative” and “analogue” also include, for example, a synthetic nucleotide or nucleoside derivative having modified base moieties and/or modified sugar moieties. Such derivatives and analogues are discussed in, for example, Scheit, *Nucleotide analogs* (John Wiley & Son, 1980) and Uhlman *et al.*, *Chemical Reviews* 90:543-584, 1990. Nucleotide analogues can also comprise modified phosphodiester linkages including phosphorothioate, phosphorodithioate, alkyl-phosphonate, phosphoranilidate, phosphoramidite linkages and the like.

[0113] In particular embodiments the labeled nucleotide may be enzymatically incorporable and enzymatically extendable. Accordingly, a linker moiety may be of sufficient length to connect the nucleotide to the compound such that the compound does not significantly interfere with the overall binding and recognition of the nucleotide by a nucleic acid replication enzyme. Thus, the linker can also comprise a spacer unit. The spacer distances, for example, the nucleotide base from a cleavage site or label.

[0114] The disclosure also encompasses polynucleotides incorporating a nucleotide described herein. Such polynucleotides may be DNA or RNA comprised respectively of

deoxyribonucleotides or ribonucleotides joined in phosphodiester linkage. Polynucleotides may comprise naturally occurring nucleotides, non-naturally occurring (or modified) nucleotides other than the labeled nucleotides described herein or any combination thereof, in combination with at least one modified nucleotide (e.g., labeled with a dye compound) as set forth herein. Polynucleotides according to the disclosure may also include non-natural backbone linkages and/or non-nucleotide chemical modifications. Chimeric structures comprised of mixtures of ribonucleotides and deoxyribonucleotides comprising at least one labeled nucleotide are also contemplated.

[0115] In some embodiments, the labeled nucleotide described herein comprises or has the structure of Formula (I):



wherein B is the nucleobase;

R⁴ is H or OH;

R⁵ is an allyl containing 3' blocking group, such as as described herein or a phosphoramidite;

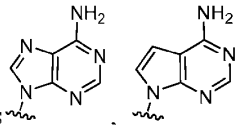
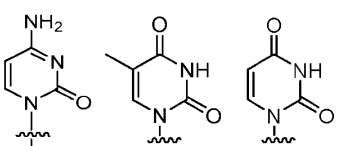
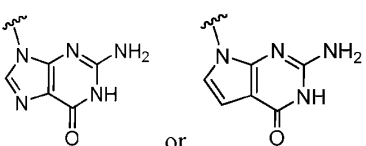
R⁶ is H, monophosphate, diphosphate, triphosphate, thiophosphate, a phosphate ester analog, a reactive phosphorous containing group, or a hydroxy protecting group;

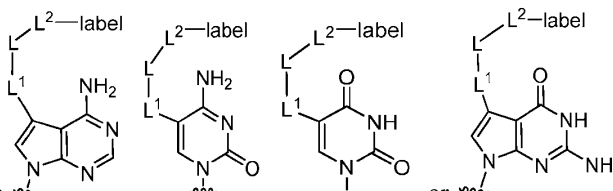
L is an allyl moiety containing linker, such as ; and each of L¹ and L² is independently an optionally present linker moiety.

[0116] In some embodiments of the nucleotide described herein, each of R^{1a}, R^{1b}, R^{2a}, R^{3a} and R^{3b} is H. In other embodiments, at least one of R^{1a}, R^{1b}, R^{2a}, R^{3a} and R^{3b} is halogen (e.g., fluoro, chloro) or unsubstituted C₁-C₆ alkyl (e.g., methyl, ethyl, isopropyl, isobutyl, or t-butyl). In some such instances, each of R^{1a} and R^{1b} is H and at least one of R^{2a}, R^{3a} and R^{3b} is unsubstituted C₁-C₆ alkyl or halogen (for example, R^{2a} is unsubstituted C₁-C₆ alkyl and each of R^{3a} and R^{3b} is H; or R^{2a} is H and one or both of R^{3a} and R^{3b} is halogen or unsubstituted C₁-C₆ alkyl). In one

embodiment, the cleavable linker or L comprises (“AOL” linker moiety).

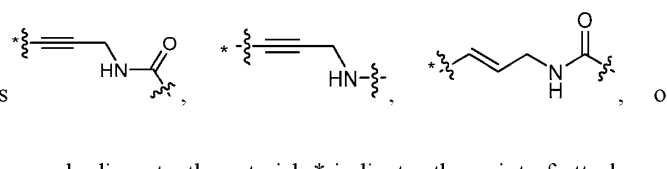
[0117] In some embodiments of the nucleotide described herein, the nucleobase (“B” in Formula (I)) is purine (adenine or guanine), a deaza purine, or a pyrimidine (e.g., cytosine, thymine or uracil). In some further embodiments, the deaza purine is 7-deaza purine (e.g., 7-deaza

adenine or 7-deaza guanine). Non-limiting examples of B comprises , , or , or optionally substituted derivatives and analogs thereof. In some further embodiments, the labeled nucleobase comprises

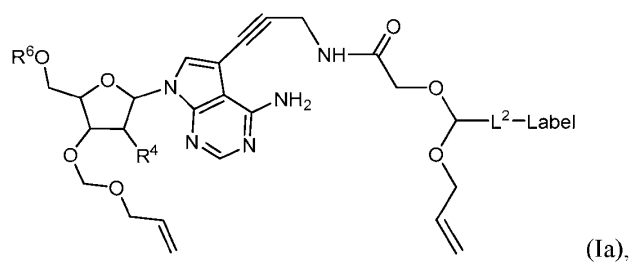
the structure .

[0118] In some other embodiments of the nucleotide described herein, R⁵ in Formula (I) is a phosphoramidite. In such embodiments, R⁶ is an acid-cleavable hydroxy protecting group which allows subsequent monomer coupling under automated synthesis conditions.

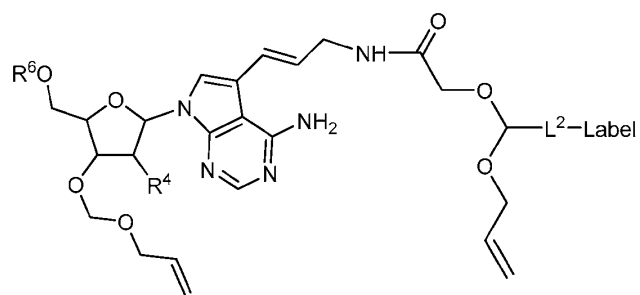
[0119] In some embodiments of the nucleotide described herein, L¹ is present and L¹ comprises a moiety selected from the group consisting of a propargylamine, a propargylamide, an allylamine, an allylamide, and optionally substituted variants thereof. In some further

embodiments, L¹ comprises . In some further embodiments, the asterisk * indicates the point of attachment of L¹ to the nucleobase (e.g., C5 position of a pyrimidine base or the C7 position of a 7-deaza purine base).

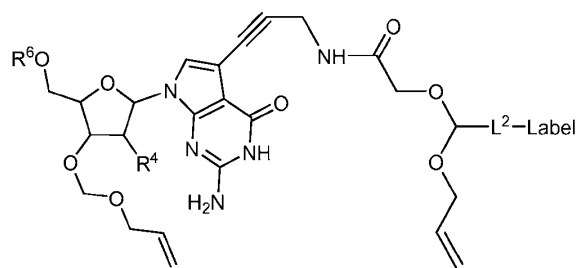
[0120] Some further embodiments of the nucleoside or nucleotide described herein include those with Formula (Ia), (Ia'), (Ib), (Ic), (Ic') or (Id):



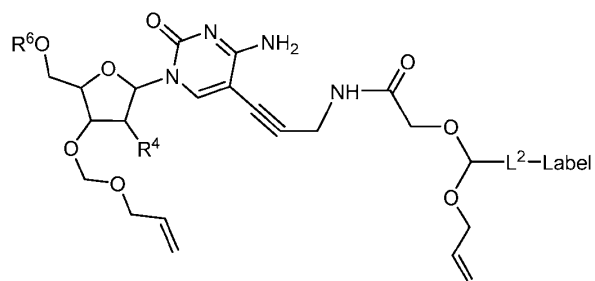
(Ia),



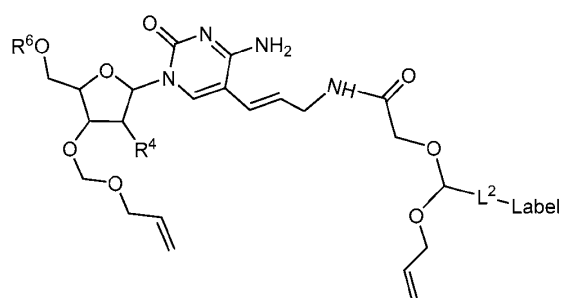
(Ia'),



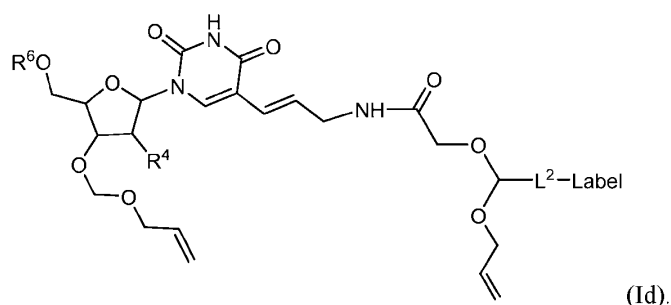
(Ib),



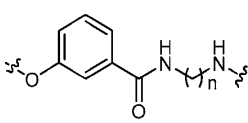
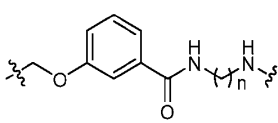
(Ic),



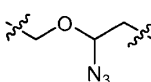
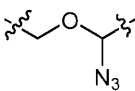
(Ic') or

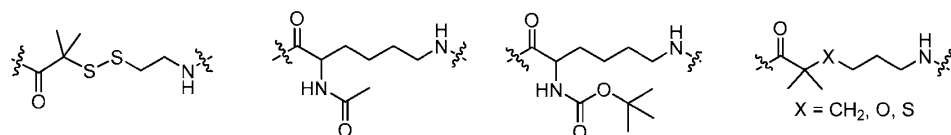


[0121] In some further embodiments of the nucleotide described herein, L^2 is present

and L^2 comprises  or , wherein n is an integer of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 and the phenyl moiety is optionally substituted. In some such embodiments, n is 5 and the phenyl moiety of L^2 is unsubstituted. In some further embodiments, R^4 is H. In some further embodiments, $-OR^6$ is a triphosphate.

[0122] In any embodiments of the nucleotide described herein, the cleavable linker or

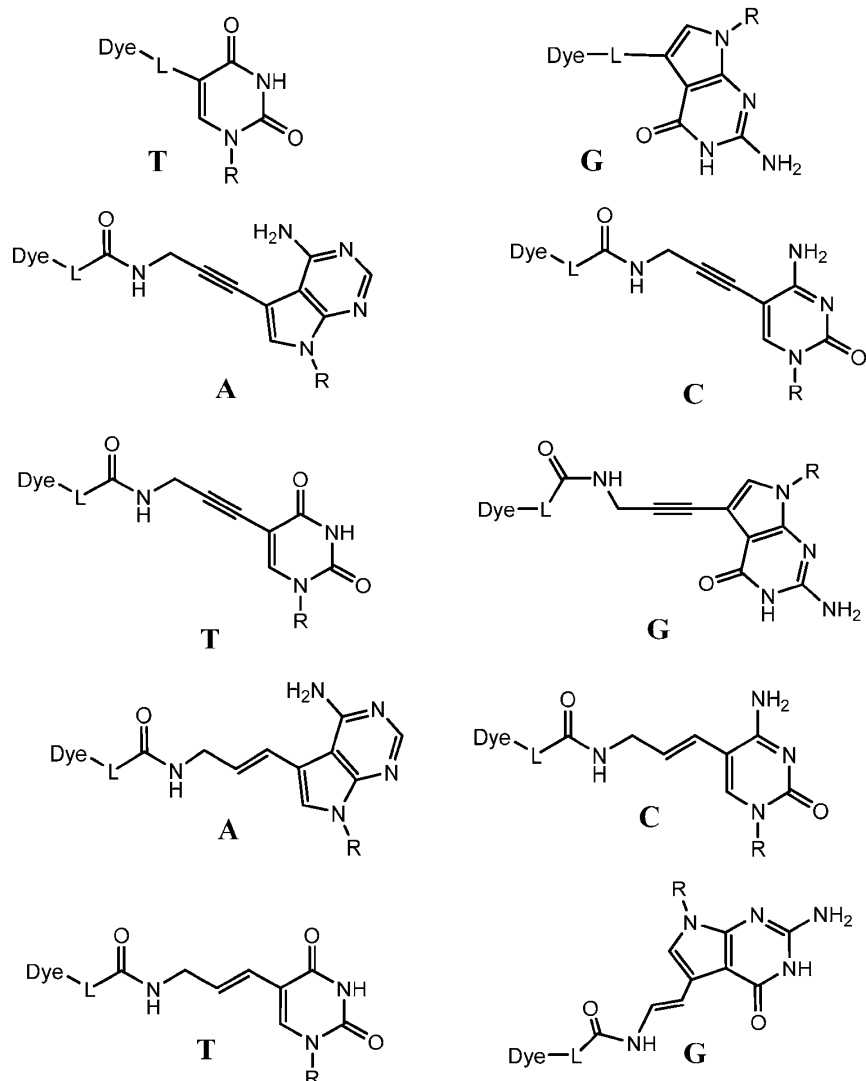
L^1/L^2 may further comprise a disulfide moiety or azido moiety (such as  or , or a combination thereof. Additional non-limiting examples of a linker moiety may be incorporated into L^1 or L^2 include:



Additional linker moieties are disclosed in WO 2004/018493 and U.S. Publication Nos. 2016/0040225 and 2021/0403500, which are herein incorporated by references.

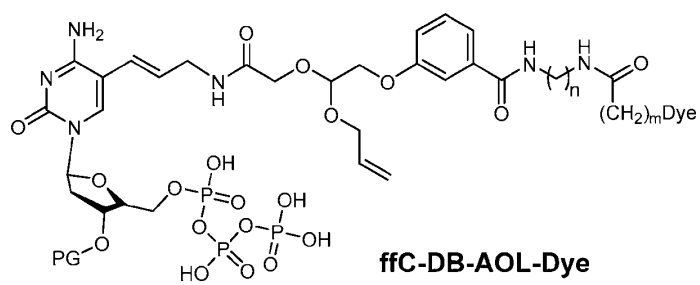
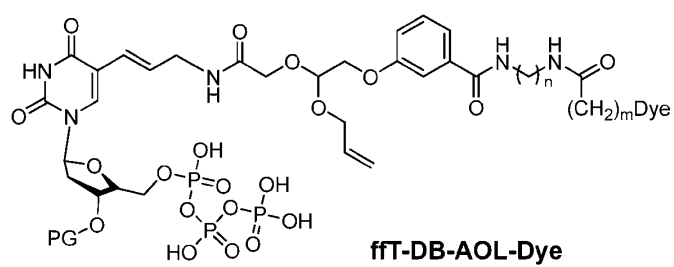
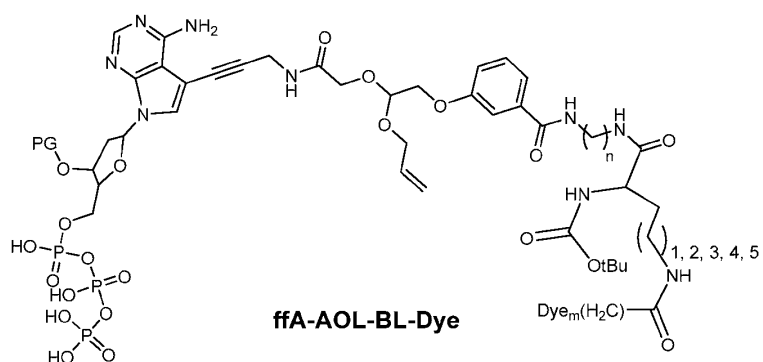
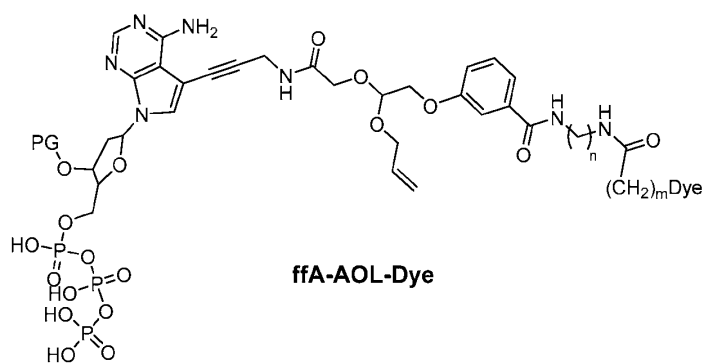
[0123] Non-limiting exemplary labeled nucleotides as described herein include:

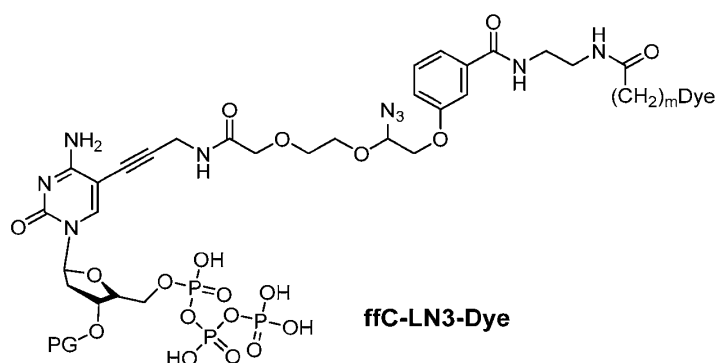




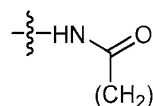
wherein L represents a cleavable linker (optionally include L^2 described herein) and R represents a ribose or deoxyribose moiety as described above, or a ribose or deoxyribose moiety with the 5' position substituted with one, two or three phosphates.

[0124] In some embodiments, non-limiting exemplary fluorescent dye conjugates are shown below:





wherein PG stands for the 3' blocking groups described herein; n is an integer of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and m is 0, 1, 2, 3, 4, or 5. In one embodiment, -O-PG is AOM. In one



embodiment, n is 5. (CH₂)_mDye refers to the connection point of the Dye with the cleavable linker as a result of a reaction between an amino group of the linker moiety and the carboxyl group of the Dye.

[0125] Various fluorescent dyes may be used in the present disclosure as detectable labels, in particularly those dyes that may be excitation by a blue light (e.g., about 450 nm to about 460 nm) or a green light (e.g., about 520 nm to about 540 nm). These dyes may also be referred to as “blue dyes” and “green dyes” respectively. Examples of various type of blue dyes, including but not limited to coumarin dyes, chromenoquinoline dyes, and bisboron containing heterocycles are disclosed in U.S. Publication Nos. 2018/0094140, 2018/0201981, 2020/0277529, 2020/0277670, 2021/0188832, 2022/0195517, 2022/0380389, and 2023/0313292, each of which is incorporated by reference in its entirety. Examples of green dyes including cyanine or polymethine dyes disclosed in International Publication Nos. WO2013/041117, WO2014/135221, WO 2016/189287, WO2017/051201 and WO2018/060482A1, each of which is incorporated by reference in its entirety.

[0126] In any embodiments of nucleotide described herein, the nucleotide comprises a 2' deoxyribose moiety (i.e., R⁴ is Formula (I) and (Ia)-(Id)) is H). In some further respect, the 2' deoxyribose contains one, two or three phosphate groups at the 5' position of the sugar ring. In some further aspect, the nucleotides described herein are nucleotide triphosphate (i.e., -OR⁶ is Formula (I) and (Ia)-(Id)) forms triphosphate).

[0127] Additional embodiments of the present disclosure relate to an oligonucleotide or a polynucleotide comprising a nucleoside or nucleotide described herein. In some such embodiments, the oligonucleotide or polynucleotide is hybridized to a template or target

polynucleotide. In some such embodiments, the template polynucleotide is immobilized on a solid support.

[0128] Additional embodiments of the present disclosure relate to a solid support comprises an array of a plurality of immobilized template or target polynucleotides and at least a portion of such immobilized template or target polynucleotides is hybridized to an oligonucleotide or a polynucleotide comprising a nucleoside or nucleotide described herein.

[0129] The present application will also be further described with reference to DNA, although the description will also be applicable to RNA, PNA, and other nucleic acids, unless otherwise indicated.

Cleavage Condition of the Cleavable Linker

[0130] In any embodiments of the nucleotides or nucleosides described herein, the 3' blocking group and the cleavable linker (and the attached label) may be removable under the same or substantially same chemical reaction conditions, for example, the 3' blocking group and the detectable label may be removed in a single chemical reaction. In other embodiments, the 3' blocking group and the detectable labeled are removed in two separate steps.

[0131] The cleavable linker described herein may be removed or cleaved under various chemical conditions. Non-limiting cleaving condition includes a palladium catalyst, such as a Pd(II) complex (e.g., Pd(OAc)₂, allylPd(II) chloride dimer [(Allyl)PdCl]₂ or Na₂PdCl₄) in the presence of a water soluble phosphine ligand, for example tris(hydroxypropyl)phosphine (THPP or THP), tris(hydroxymethyl)phosphine, and/or tris(2-carboxyethyl)phosphine (TCEP), with or without the presence of a reducing agent. Non-limiting cleaving condition includes a nickel catalyst, such as a Ni(II) compound (NiCl₂) in the presence of a phosphine ligand, for example tris(hydroxypropyl)phosphine, tris(hydroxymethyl)phosphine, and/or tris(2-carboxyethyl)phosphine. In some embodiments, the 3' blocking group may be cleaved under the same or substantially the same cleavage condition as that for the cleavable linker.

Compatibility with Linearization

[0132] In order to maximize the throughput of nucleic acid sequencing reactions it is advantageous to be able to sequence multiple template molecules in parallel. Parallel processing of multiple templates can be achieved with the use of nucleic acid array technology. These arrays typically consist of a high-density matrix of polynucleotides immobilized onto a solid support material.

[0133] WO 98/44151 and WO 00/18957 both describe methods of nucleic acid amplification which allow amplification products to be immobilized on a solid support in order to

form arrays comprised of clusters or “colonies” formed from a plurality of identical immobilized polynucleotide strands and a plurality of identical immobilized complementary strands. Arrays of this type are referred to herein as “clustered arrays.” The nucleic acid molecules present in DNA colonies on the clustered arrays prepared according to these methods can provide templates for sequencing reactions, for example as described in WO 98/44152. The products of solid-phase amplification reactions such as those described in WO 98/44151 and WO 00/18957 are so-called “bridged” structures formed by annealing of pairs of immobilized polynucleotide strands and immobilized complementary strands, both strands being attached to the solid support at the 5' end. In order to provide more suitable templates for nucleic acid sequencing, it is preferred to remove substantially all or at least a portion of one of the immobilized strands in the “bridged” structure in order to generate a template which is at least partially single-stranded. The portion of the template which is single-stranded will thus be available for hybridization to a sequencing primer. The process of removing all or a portion of one immobilized strand in a “bridged” double-stranded nucleic acid structure is referred to as “linearization.” There are various ways for linearization, including but not limited to enzymatic cleavage, photo-chemical cleavage, or chemical cleavage. Non-limiting examples of linearization methods are disclosed in PCT Publication No. WO 2007/010251, U.S. Patent Publication No. 2009/0088327, U.S. Patent Publication No. 2009/0118128, and U.S. Publication No. 2019/0352327, which are incorporated by reference in their entireties.

[0134] In some embodiments, the condition for the removal of the 3' blocking group and/or the cleavable linker is also compatible with the linearization processes, for example, a chemical linearization process which comprises the use of a Pd complex and a phosphine. In some embodiments, the Pd complex is a Pd(II) complex (e.g., Pd(OAc)₂, [(Allyl)PdCl]₂, K₂PdCl₄ or Na₂PdCl₄), which generates Pd(0) in situ in the presence of a water soluble phosphine described herein, without or without the presence of a reducing agent.

Embodiments and Alternatives of Sequencing-By-Synthesis

[0135] Alternatively, the sequencing methods described herein may also be carried out using unlabeled nucleotides and affinity reagents containing a fluorescent dye described herein. For example, one, two, three or each of the four different types of nucleotides (e.g., dATP, dCTP, dGTP and dTTP or dUTP) in the incorporation mixture of step (a) may be unlabeled. Each of the four types of nucleotides (e.g., dNTPs) has a 3' blocking group to ensure that only a single base can be added by a polymerase to the 3' end of the primer polynucleotide. After incorporation of an unlabeled nucleotide in step (b), the remaining unincorporated nucleotides are washed away. An affinity reagent is then introduced that specifically recognizes and binds to the incorporated

dNTP to provide a labeled extension product comprising the incorporated dNTP. Uses of unlabeled nucleotides and affinity reagents in sequencing by synthesis have been disclosed in WO 2018/129214 and WO 2020/097607. In addition, post-incorporation labeling sequencing methods have been described in U.S. Publication 2023/0383342 A1 and U.S. Ser. No. 63/579897, each of which is incorporated by reference. A modified sequencing method of the present disclosure using unlabeled nucleotides may include the following steps:

(a') contacting a solid support with sequencing primers under hybridization conditions, wherein the solid support comprises a plurality of target polynucleotides immobilized thereon; and the sequencing primers are complementary to at least a portion of the target polynucleotides;

(b') contacting the solid support with a first aqueous solution comprising DNA polymerase and one or more of four different types of nucleotides under conditions suitable for DNA polymerase-mediated primer extension, wherein each of the nucleotides comprises a 3' blocking group comprising an unsubstituted or substituted allyl moiety as described herein;

(c') contacting the extended copy polynucleotides with a set of affinity reagents under conditions wherein one affinity reagent binds specifically to the incorporated unlabeled nucleotides to provide labeled extended copy polynucleotides;

(d') imaging the solid support and performing one or more fluorescent measurements of the extended copy polynucleotides; and

(e') removing the 3' blocking group of the incorporated nucleotides in a second aqueous solution comprising a transition metal catalyst and one or more reducing agents, the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing tris(substituted C₁-C₆ alkyl)phosphine, and wherein the one or more reducing agents comprise a boron-containing reducing agent.

[0136] In some embodiments of the modified sequencing method described herein, the method further comprises removing the affinity reagents from the incorporated nucleotides. In still further embodiments, the 3' blocking group and the affinity reagent are removed in the same reaction. In some embodiments, the method further comprises a step (f') washing the solid support with a third aqueous wash solution. In further embodiments, steps (b') through (f') are repeated at least 50, 100, 150, 200, 250, 300, 350, 400, 450 or 500 cycles to determine the target polynucleotide sequences. In some embodiments, the set of affinity reagents may comprise a first affinity reagent that binds specifically to the first type of nucleotide, a second affinity reagent that binds specifically to the second type of nucleotide, and a third affinity reagent that binds specifically to the third type of nucleotide. In some further embodiments, each of the first, second

and the third affinity reagents comprises a detectable labeled that is spectrally distinguishable. In some embodiments, the affinity reagents may include protein tags, antibodies (including but not limited to binding fragments of antibodies, single chain antibodies, bispecific antibodies, and the like), aptamers, knottins, affimers, or any other known agent that binds an incorporated nucleotide with a suitable specificity and affinity. In one embodiment, at least one affinity reagent is an antibody or a protein tag. In another embodiment, at least one of the first type, the second type, and the third type of affinity reagents is an antibody or a protein tag comprising one or more detectable labels (e.g., multiple copies of the same detectable label), wherein the detectable label is or comprises a bis-boron dye moiety described herein.

[0137] Some embodiments include pyrosequencing techniques. Pyrosequencing detects the release of inorganic pyrophosphate (PPi) as particular nucleotides are incorporated into the nascent strand (Ronaghi, M., Karamohamed, S., Pettersson, B., Uhlen, M. and Nyren, P. (1996) "Real-time DNA sequencing using detection of pyrophosphate release." *Analytical Biochemistry* 242(1), 84-9; Ronaghi, M. (2001) "Pyrosequencing sheds light on DNA sequencing." *Genome Res.* 11(1), 3-11; Ronaghi, M., Uhlen, M. and Nyren, P. (1998) "A sequencing method based on real-time pyrophosphate." *Science* 281(5375), 363; U.S. Pat. Nos. 6,210,891; 6,258,568 and 6,274,320, the disclosures of which are incorporated herein by reference in their entireties). In pyrosequencing, released PPi can be detected by being immediately converted to adenosine triphosphate (ATP) by ATP sulfurase, and the level of ATP generated is detected via luciferase-produced photons. The nucleic acids to be sequenced can be attached to features in an array and the array can be imaged to capture the chemiluminescent signals that are produced due to incorporation of a nucleotides at the features of the array. An image can be obtained after the array is treated with a particular nucleotide type (e.g., A, T, C or G). Images obtained after addition of each nucleotide type will differ with regard to which features in the array are detected. These differences in the image reflect the different sequence content of the features on the array. However, the relative locations of each feature will remain unchanged in the images. The images can be stored, processed and analyzed using the methods set forth herein. For example, images obtained after treatment of the array with each different nucleotide type can be handled in the same way as exemplified herein for images obtained from different detection channels for reversible terminator-based sequencing methods.

[0138] In another exemplary type of SBS, cycle sequencing is accomplished by stepwise addition of reversible terminator nucleotides containing, for example, a cleavable or photobleachable dye label as described, for example, in WO 04/018497 and U.S. Pat. No. 7,057,026, the disclosures of which are incorporated herein by reference. This approach is being commercialized by Solexa (now Illumina, Inc.), and is also described in WO 91/06678 and WO

07/123,744, each of which is incorporated herein by reference. The availability of fluorescently-labeled terminators in which both the termination can be reversed, and the fluorescent label cleaved facilitates efficient cyclic reversible termination (CRT) sequencing. Polymerases can also be co-engineered to efficiently incorporate and extend from these modified nucleotides.

[0139] Preferably in reversible terminator-based sequencing embodiments, the labels do not substantially inhibit extension under SBS reaction conditions. However, the detection labels can be removable, for example, by cleavage or degradation. Images can be captured following incorporation of labels into arrayed nucleic acid features. In particular embodiments, each cycle involves simultaneous delivery of four different nucleotide types to the array and each nucleotide type has a spectrally distinct label. Four images can then be obtained, each using a detection channel that is selective for one of the four different labels. Alternatively, different nucleotide types can be added sequentially, and an image of the array can be obtained between each addition step. In such embodiments each image will show nucleic acid features that have incorporated nucleotides of a particular type. Different features will be present or absent in the different images due the different sequence content of each feature. However, the relative position of the features will remain unchanged in the images. Images obtained from such reversible terminator-SBS methods can be stored, processed and analyzed as set forth herein. Following the image capture step, labels can be removed, and reversible terminator moieties can be removed for subsequent cycles of nucleotide addition and detection. Removal of the labels after they have been detected in a particular cycle and prior to a subsequent cycle can provide the advantage of reducing background signal and crosstalk between cycles. Examples of useful labels and removal methods are set forth below.

[0140] Some embodiments can utilize detection of four different nucleotides using fewer than four different labels. For example, SBS can be performed utilizing methods and systems described in the incorporated materials of U.S. Pub. No. 2013/0079232. As a first example, a pair of nucleotide types can be detected at the same wavelength, but distinguished based on a difference in intensity for one member of the pair compared to the other, or based on a change to one member of the pair (e.g. via chemical modification, photochemical modification or physical modification) that causes apparent signal to appear or disappear compared to the signal detected for the other member of the pair. As a second example, three of four different nucleotide types can be detected under particular conditions while a fourth nucleotide type lacks a label that is detectable under those conditions, or is minimally detected under those conditions (e.g., minimal detection due to background fluorescence, etc.). Incorporation of the first three nucleotide types into a nucleic acid can be determined based on presence of their respective signals and incorporation of the fourth nucleotide type into the nucleic acid can be determined based on

absence or minimal detection of any signal. As a third example, one nucleotide type can include label(s) that are detected in two different channels, whereas other nucleotide types are detected in no more than one of the channels. The aforementioned three exemplary configurations are not considered mutually exclusive and can be used in various combinations. An exemplary embodiment that combines all three examples, is a fluorescent-based SBS method that uses a first nucleotide type that is detected in a first channel (e.g. dATP having a label that is detected in the first channel when excited by a first excitation wavelength), a second nucleotide type that is detected in a second channel (e.g. dCTP having a label that is detected in the second channel when excited by a second excitation wavelength), a third nucleotide type that is detected in both the first and the second channel (e.g. dTTP having at least one label that is detected in both channels when excited by the first and/or second excitation wavelength) and a fourth nucleotide type that lacks a label that is not, or minimally, detected in either channel (e.g. dGTP having no label).

[0141] Further, as described in the incorporated materials of U.S. Pub. No. 2013/0079232, sequencing data can be obtained using a single channel. In such so-called one-dye sequencing approaches, the first nucleotide type is labeled but the label is removed after the first image is generated, and the second nucleotide type is labeled only after a first image is generated. The third nucleotide type retains its label in both the first and second images, and the fourth nucleotide type remains unlabeled in both images.

[0142] Some embodiments can utilize sequencing by ligation techniques. Such techniques utilize DNA ligase to incorporate oligonucleotides and identify the incorporation of such oligonucleotides. The oligonucleotides typically have different labels that are correlated with the identity of a particular nucleotide in a sequence to which the oligonucleotides hybridize. As with other SBS methods, images can be obtained following treatment of an array of nucleic acid features with the labeled sequencing reagents. Each image will show nucleic acid features that have incorporated labels of a particular type. Different features will be present or absent in the different images due the different sequence content of each feature, but the relative position of the features will remain unchanged in the images. Images obtained from ligation-based sequencing methods can be stored, processed and analyzed as set forth herein. Exemplary SBS systems and methods which can be utilized with the methods and systems described herein are described in U.S. Pat. Nos. 6,969,488, 6,172,218, and 6,306,597, the disclosures of which are incorporated herein by reference in their entireties.

[0143] Some embodiments can utilize nanopore sequencing (Deamer, D. W. & Akeson, M. "Nanopores and nucleic acids: prospects for ultrarapid sequencing." *Trends Biotechnol.* 18, 147-151 (2000); Deamer, D. and D. Branton, "Characterization of nucleic acids by nanopore analysis," *Acc. Chem. Res.* 35:817-825 (2002); Li, J., M. Gershow, D. Stein, E.

Brandin, and J. A. Golovchenko, "DNA molecules and configurations in a solid-state nanopore microscope," *Nat. Mater.* 2:611-615 (2003), the disclosures of which are incorporated herein by reference in their entireties). In such embodiments, the target nucleic acid passes through a nanopore. The nanopore can be a synthetic pore or biological membrane protein, such as α -hemolysin. As the target nucleic acid passes through the nanopore, each base-pair can be identified by measuring fluctuations in the electrical conductance of the pore. (U.S. Pat. No. 7,001,792; Soni, G. V. & Meller, "A. Progress toward ultrafast DNA sequencing using solid-state nanopores," *Clin. Chem.* 53, 1996-2001 (2007); Healy, K. "Nanopore-based single-molecule DNA analysis," *Nanomed.* 2, 459-481 (2007); Cockroft, S. L., Chu, J., Amorin, M. & Ghadiri, M. R. "A single-molecule nanopore device detects DNA polymerase activity with single-nucleotide resolution," *J. Am. Chem. Soc.* 130, 818-820 (2008), the disclosures of which are incorporated herein by reference in their entireties). Data obtained from nanopore sequencing can be stored, processed and analyzed as set forth herein. In particular, the data can be treated as an image in accordance with the exemplary treatment of optical images and other images that is set forth herein.

[0144] Some other embodiments of sequencing method involve the use the 3' blocked nucleotide described herein in nanoball sequencing technique, such as those described in U.S. Patent No. 9,222,132, the disclosure of which is incorporated by reference. Through the process of rolling circle amplification (RCA), a large number of discrete DNA nanoballs may be generated. The nanoball mixture is then distributed onto a patterned slide surface containing features that allow a single nanoball to associate with each location. In DNA nanoball generation, DNA is fragmented and ligated to the first of four adapter sequences. The template is amplified, circularized and cleaved with a type II endonuclease. A second set of adapters is added, followed by amplification, circularization and cleavage. This process is repeated for the remaining two adapters. The final product is a circular template with four adapters, each separated by a template sequence. Library molecules undergo a rolling circle amplification step, generating a large mass of concatemers called DNA nanoballs, which are then deposited on a flow cell. Goodwin *et al.*, "Coming of age: ten years of next-generation sequencing technologies," *Nat Rev Genet.* 2016;17(6):333-51.

[0145] Some embodiments can utilize methods involving the real-time monitoring of DNA polymerase activity. Nucleotide incorporations can be detected through fluorescence resonance energy transfer (FRET) interactions between a fluorophore-bearing polymerase and γ -phosphate-labeled nucleotides as described, for example, in U.S. Pat. Nos. 7,329,492 and 7,211,414, both of which are incorporated herein by reference, or nucleotide incorporations can be detected with zero-mode waveguides as described, for example, in U.S. Pat. No. 7,315,019, which is incorporated herein by reference, and using fluorescent nucleotide analogs and

engineered polymerases as described, for example, in U.S. Pat. No. 7,405,281 and U.S. Pub. No. 2008/0108082, both of which are incorporated herein by reference. The illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of fluorescently labeled nucleotides can be observed with low background (Levene, M. J. *et al.* "Zero-mode waveguides for single-molecule analysis at high concentrations," *Science* 299, 682-686 (2003); Lundquist, P. M. *et al.* "Parallel confocal detection of single molecules in real time," *Opt. Lett.* 33, 1026-1028 (2008); Korlach, J. *et al.* "Selective aluminum passivation for targeted immobilization of single DNA polymerase molecules in zero-mode waveguide nano structures," *Proc. Natl. Acad. Sci. USA* 105, 1176-1181 (2008), the disclosures of which are incorporated herein by reference in their entireties). Images obtained from such methods can be stored, processed and analyzed as set forth herein.

[0146] Some SBS embodiments include detection of a proton released upon incorporation of a nucleotide into an extension product. For example, sequencing based on detection of released protons can use an electrical detector and associated techniques that are commercially available from Ion Torrent (Guilford, CT, a Life Technologies subsidiary) or sequencing methods and systems described in U.S. Pub. Nos. 2009/0026082; 2009/0127589; 2010/0137143; and 2010/0282617, all of which are incorporated herein by reference. Methods set forth herein for amplifying target nucleic acids using kinetic exclusion can be readily applied to substrates used for detecting protons. More specifically, methods set forth herein can be used to produce clonal populations of amplicons that are used to detect protons.

[0147] The above SBS methods can be advantageously carried out in multiplex formats such that multiple different target nucleic acids are manipulated simultaneously. In particular embodiments, different target nucleic acids can be treated in a common reaction vessel or on a surface of a particular substrate. This allows convenient delivery of sequencing reagents, removal of unreacted reagents and detection of incorporation events in a multiplex manner. In embodiments using surface-bound target nucleic acids, the target nucleic acids can be in an array format. In an array format, the target nucleic acids can be typically bound to a surface in a spatially distinguishable manner. The target nucleic acids can be bound by direct covalent attachment, attachment to a bead or other particle or binding to a polymerase or other molecule that is attached to the surface. The array can include a single copy of a target nucleic acid at each site (also referred to as a feature) or multiple copies having the same sequence can be present at each site or feature. Multiple copies can be produced by amplification methods such as, bridge amplification or emulsion PCR as described in further detail below.

[0148] The methods set forth herein can use arrays having features at any of a variety of densities including, for example, at least about 10 features/cm², 100 features/cm², 500

features/cm², 1,000 features/cm², 5,000 features/cm², 10,000 features/cm², 50,000 features/cm², 100,000 features/cm², 1,000,000 features/cm², 5,000,000 features/cm², or higher.

[0149] An advantage of the methods set forth herein is that they provide for rapid and efficient detection of a plurality of target nucleic acid in parallel. Accordingly, the present disclosure provides integrated systems capable of preparing and detecting nucleic acids using techniques known in the art such as those exemplified above. Thus, an integrated system of the present disclosure can include fluidic components capable of delivering amplification reagents and/or sequencing reagents to one or more immobilized DNA fragments, the system comprising components such as pumps, valves, reservoirs, fluidic lines and the like. A flow cell can be configured and/or used in an integrated system for detection of target nucleic acids. Exemplary flow cells are described, for example, in U.S. Pub. No. 2010/0111768 and U.S. Patent Appl. No. 13/273,666, each of which is incorporated herein by reference. As exemplified for flow cells, one or more of the fluidic components of an integrated system can be used for an amplification method and for a detection method. Taking a nucleic acid sequencing embodiment as an example, one or more of the fluidic components of an integrated system can be used for an amplification method set forth herein and for the delivery of sequencing reagents in a sequencing method such as those exemplified above. Alternatively, an integrated system can include separate fluidic systems to carry out amplification methods and to carry out detection methods. Examples of integrated sequencing systems that are capable of creating amplified nucleic acids and also determining the sequence of the nucleic acids include, without limitation, the MiSeq™ platform (Illumina, Inc., San Diego, CA) and devices described in U.S. Patent Appl. No. 13/273,666, which is incorporated herein by reference.

[0150] Arrays in which polynucleotides have been directly attached to silica-based supports are those for example disclosed in WO 00/06770 (incorporated herein by reference), wherein polynucleotides are immobilized on a glass support by reaction between a pendant epoxide group on the glass with an internal amino group on the polynucleotide. In addition, polynucleotides can be attached to a solid support by reaction of a sulfur-based nucleophile with the solid support, for example, as described in WO 2005/047301 (incorporated herein by reference). A still further example of solid-supported template polynucleotides is where the template polynucleotides are attached to hydrogel supported upon silica-based or other solid supports, for example, as described in WO 00/31148, WO 01/01143, WO 02/12566, WO 03/014392, U.S. Pat. No. 6,465,178 and WO 00/53812, each of which is incorporated herein by reference.

[0151] A particular surface to which template polynucleotides may be immobilized is a polyacrylamide hydrogel. Polyacrylamide hydrogels are described in the references cited above

and in WO 2005/065814, which is incorporated herein by reference. Specific hydrogels that may be used include those described in WO 2005/065814 and U.S. Pub. No. 2014/0079923. In one embodiment, the hydrogel is PAZAM (poly(N-(5-azidoacetamidylpentyl) acrylamide-co-acrylamide)).

[0152] DNA template molecules can be attached to beads or microparticles, for example, as described in U.S. Pat. No. 6,172,218 (which is incorporated herein by reference). Attachment to beads or microparticles can be useful for sequencing applications. Bead libraries can be prepared where each bead contains different DNA sequences. Exemplary libraries and methods for their creation are described in *Nature*, 437, 376-380 (2005); *Science*, 309, 5741, 1728-1732 (2005), each of which is incorporated herein by reference. Sequencing of arrays of such beads using nucleotides set forth herein is within the scope of the disclosure.

[0153] Templates that are to be sequenced may form part of an “array” on a solid support, in which case the array may take any convenient form. Thus, the method of the disclosure is applicable to all types of high-density arrays, including single-molecule arrays, clustered arrays, and bead arrays. Labeled nucleotides of the present disclosure may be used for sequencing templates on essentially any type of array, including but not limited to those formed by immobilization of nucleic acid molecules on a solid support.

[0154] However, labeled nucleotides of the disclosure are particularly advantageous in the context of sequencing of clustered arrays. In clustered arrays, distinct regions on the array (often referred to as sites, or features) comprise multiple polynucleotide template molecules. Generally, the multiple polynucleotide molecules are not individually resolvable by optical means and are instead detected as an ensemble. Depending on how the array is formed, each site on the array may comprise multiple copies of one individual polynucleotide molecule (e.g., the site is homogenous for a particular single- or double-stranded nucleic acid species) or even multiple copies of a small number of different polynucleotide molecules (e.g., multiple copies of two different nucleic acid species). Clustered arrays of nucleic acid molecules may be produced using techniques generally known in the art. By way of example, WO 98/44151 and WO 00/18957, each of which is incorporated herein, describe methods of amplification of nucleic acids wherein both the template and amplification products remain immobilized on a solid support in order to form arrays comprised of clusters or “colonies” of immobilized nucleic acid molecules. The nucleic acid molecules present on the clustered arrays prepared according to these methods are suitable templates for sequencing using the nucleotides labeled with dye compounds of the disclosure.

[0155] The labeled nucleotides of the present disclosure are also useful in sequencing of templates on single molecule arrays. The term “single molecule array” (“SMA”) as used herein

refers to a population of polynucleotide molecules, distributed (or arrayed) over a solid support, wherein the spacing of any individual polynucleotide from all others of the population is such that it is possible to individually resolve the individual polynucleotide molecules. The target nucleic acid molecules immobilized onto the surface of the solid support can thus be capable of being resolved by optical means in some embodiments. This means that one or more distinct signals, each representing one polynucleotide, will occur within the resolvable area of the particular imaging device used.

[0156] Single molecule detection may be achieved wherein the spacing between adjacent polynucleotide molecules on an array is at least 100 nm, more particularly at least 250 nm, still more particularly at least 300 nm, even more particularly at least 350 nm. Thus, each molecule is individually resolvable and detectable as a single molecule fluorescent point, and fluorescence from said single molecule fluorescent point also exhibits single step photobleaching.

[0157] The terms “individually resolved” and “individual resolution” are used herein to specify that, when visualized, it is possible to distinguish one molecule on the array from its neighboring molecules. Separation between individual molecules on the array will be determined, in part, by the particular technique used to resolve the individual molecules. The general features of single molecule arrays will be understood by reference to published applications WO 00/06770 and WO 01/57248, each of which is incorporated herein by reference. Although one use of the nucleotides of the disclosure is in sequencing-by-synthesis reactions, the utility of the nucleotides is not limited to such methods. In fact, the nucleotides may be used advantageously in any sequencing methodology which requires detection of fluorescent labels attached to nucleotides incorporated into a polynucleotide.

[0158] In particular, the labeled nucleotides of the disclosure may be used in automated fluorescent sequencing protocols, particularly fluorescent dye-terminator cycle sequencing based on the chain termination sequencing method of Sanger and co-workers. Such methods generally use enzymes and cycle sequencing to incorporate fluorescently labeled dideoxynucleotides in a primer extension sequencing reaction. So-called Sanger sequencing methods, and related protocols (Sanger-type), utilize randomized chain termination with labeled dideoxynucleotides.

[0159] Thus, the present disclosure also encompasses labeled nucleotides which are dideoxynucleotides lacking hydroxy groups at both of the 3' and 2' positions, such dideoxynucleotides being suitable for use in Sanger type sequencing methods and the like.

[0160] Labeled nucleotides of the present disclosure incorporating 3' blocking groups, it will be recognized, may also be of utility in Sanger methods and related protocols since the same effect achieved by using dideoxy nucleotides may be achieved by using nucleotides having 3'-OH

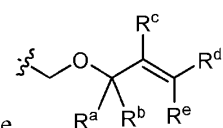
blocking groups: both prevent incorporation of subsequent nucleotides. Where nucleotides according to the present disclosure, and having a 3' blocking group are to be used in Sanger-type sequencing methods it will be appreciated that the dye compounds or detectable labels attached to the nucleotides need not be connected via cleavable linkers, since in each instance where a labeled nucleotide of the disclosure is incorporated; no nucleotides need to be subsequently incorporated and thus the label need not be removed from the nucleotide.

Kits

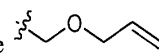
[0161] The present disclosure also provides kits for use with a sequencing apparatus, comprising: an aqueous cleavage mixture comprising a transition metal catalyst and one or more reducing agents, wherein the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand. In some embodiments, the one or more reducing agents comprise a boron-containing reducing agent described herein. In some embodiments, the transition metal catalyst may be a Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the water-soluble non-reducing phosphine. In a further embodiment, the Pd(0) catalyst can be generated *in situ* from the palladium complex and the water-soluble non-reducing tris(substituted C₁-C₆ alkyl)phosphine described herein in connection with the method of sequencing. In yet a further embodiment, the palladium complex may include [Pd(Allyl)Cl]₂, Na₂PdCl₄, K₂PdCl₄, Li₂PdCl₄, [Pd(Allyl)(THPP)]Cl, [Pd(Allyl)(THPP)]₂Cl, Pd(CH₃CN)₂Cl₂, Pd(OAc)₂, Pd(PPh₃)₄, Pd(dba)₂, Pd(Acac)₂, PdCl₂(COD), Pd(TFA)₂, Na₂PdBr₄, K₂PdBr₄, PdCl₂, PdBr₂, or Pd(NO₃)₂, or combinations thereof. In yet a further embodiment, the palladium complex may include [Pd(Allyl)Cl]₂ or Na₂PdCl₄. In some embodiments, the water soluble non-reducing phosphine comprises or is tris(substituted C₁-C₆ alkyl)phosphine or tris(substituted C₆-C₁₀ aryl)phosphine, or a combination thereof. In some embodiments, the tris(substituted C₁-C₆ alkyl)phosphine is a tris(carboxy substituted C₁-C₆ alkyl)phosphine. In a further embodiment, the tris(substituted C₁-C₆ alkyl)phosphine is TCEP. In some other embodiments, the tris(substituted C₁-C₆ alkyl)phosphine is DIPA or diBuPpropSO₃. In some embodiments, the molar ratio of the palladium complex and the water soluble non-reducing phosphine is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10. In some other embodiments, the transition metal catalyst is a Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the N,N-bidentate non-phosphine ligand. In some embodiments, the molar ratio of the palladium complex and the N,N-bidentate non-phosphine ligand is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10. In some embodiments, the boron-containing reducing agent is NH₃BH₃ or B₂(OH)₄. In

some embodiments, the pH of the aqueous cleavage mixture is from about 7.0 to about 10, or from about 7.5 to about 9.5. Other suitable non-reducing water soluble phosphines and reducing agents may also be used, such as those described above in connection with the method of sequencing.

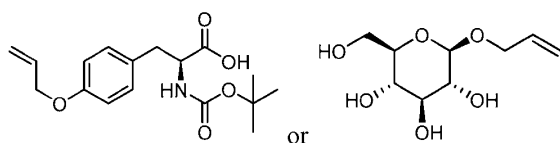
[0162] Some embodiments further comprise an incorporation mixture, where the incorporation mixture comprises one or more of four different types of nucleotides (e.g., four different types of nucleotides from A, T, C and G or U; dATP, dTTP, dCTP and dGTP or dUTP), wherein each of the nucleotides has a 3' blocking group described herein. In further embodiments, the incorporation mixture may comprise at least one Pd(0) scavenger as described herein. In



further embodiments, the 3' blocking group of the nucleotide has the structure attached to the 3' oxygen of the nucleotide, wherein each of R^a , R^b , R^c , R^d and R^e is independently H, halogen, unsubstituted or substituted C_1 - C_6 alkyl, or C_1 - C_6 haloalkyl. In yet a further embodiment, the 3' blocking group of the nucleotide has the structure attached to the 3' oxygen of the nucleotide.



In some further embodiments, the Pd(0) scavenger includes one or more allyl moieties selected from the group consisting of $-O$ -allyl, $-S$ -allyl, $-NR$ -allyl, and $-N^+RR'$ -allyl, and combinations thereof, wherein R is H, unsubstituted or substituted C_1 - C_6 alkyl, unsubstituted or substituted C_2 - C_6 alkenyl, unsubstituted or substituted C_2 - C_6 alkynyl, unsubstituted or substituted C_6 - C_{10} aryl, unsubstituted or substituted 5 to 10 membered heteroaryl, unsubstituted or substituted C_3 - C_{10} carbocyclyl, or unsubstituted or substituted 5 to 10 membered heterocyclyl; and R' is H, unsubstituted C_1 - C_6 alkyl or substituted C_1 - C_6 alkyl. In a further embodiment, the Pd(0) scavenger comprising one or more $-O$ -allyl moieties is



, or a salt thereof. In a further embodiment, the Pd(0) scavenger comprising one or more one or more $-NR$ -allyl or $-N^+RR'$ -allyl moieties is Z^- , wherein Z^- is Cl^- or F^- . In a further embodiment, the incorporation mixture is in a lyophilized form.

[0163] Some embodiments of the kit further comprise an aqueous wash solution, or a composition that is reconstitutable into an aqueous wash solution. In some embodiments, the aqueous wash solution comprises at least one Pd(II) scavenger as described herein.

[0164] The present disclosure also provides for a cartridge for use with a sequencing apparatus, comprising a plurality of chambers, where one or more of the plurality of chambers is

for use with the kit comprising the aqueous cleavage mixture described herein. For example, the cartridge may contain two or more separate chambers, one chamber contains the aqueous cleavage mixture described herein, and another chamber contains the incorporation mixture described herein.

EXAMPLES

[0165] Additional embodiments are disclosed in further detail in the following examples, which are not in any way intended to limit the scope of the claims.

Example 1. Efficiency of TCEP with a Reducing Agent for Removing 3' Blocking Group

[0166] The efficiency of TCEP as a non-reducing ligand was tested with and without the presence of a reducing agent NH_3BH_3 against THPP, a reducing ligand in solution assays. The substrate used for cleavage was a fully functionalized A nucleotide with 3' AOM blocking group and the pH of the cleavage mixture in all conditions was approximately 9.5. The Pd(0) catalyst was generated from Na_2PdCl_4 , a Pd(II) complex.

[0167] As shown in **FIG. 1**, the cleavage mixture with TCEP and NH_3BH_3 exhibited a higher cleavage rate than that of THPP, reaching close to 100% cleavage at 1 minute. The cleavage rate of TCEP without a reducing reagent was approximately 0, confirming that TCEP by itself could not transform Pd(II) to the active Pd(0) form.

Example 2. Efficiency of Pd Cleavage Mixture at Neutral pH

[0168] The efficiency of cleavage mixture containing TCEP and NH_3BH_3 at a pH of about 7 was tested against a standard Pd cleavage mixture UCM) containing THPP at two different pH: about 7 and about 9.5 in solution assays. UCM is a Pd cleavage mixture used in several Illumina's SBS platforms, and typically has a pH of 9.5. The substrate used for cleavage was a fully functionalized A nucleotide with 3' AOM blocking group. The Pd(0) catalyst was generated from Na_2PdCl_4 .

[0169] As shown in **FIG. 2**, UCM at pH 9.5 achieved about 100% cleavage by 5 minutes. TCEP at pH 7 was able to achieve about 90% cleavage at 10 minutes, and about 100% cleavage at 30 minutes. UCM at pH 7 was not as quick, achieving only about 75% cleavage at 50 minutes. The results indicate that TCEP may be suitable for inclusion in reagents where a neutral pH is desirable.

Example 3. Efficiency of TCEP with 50% Pd Concentration

[0170] The efficiency of TCEP in the presence of a reducing agent $B_2(OH)_4$ in a cleavage mixture having 50% of Pd concentration compared to the standard UCM was tested in the presence of a reducing agent $B_2(OH)_4$ against UCM in solution assays, with 50% and 100% Pd. UCM is typically used with 100% Pd. The substrate used for cleavage was a fully functionalized A nucleotide with 3' AOM blocking group and the pH of all conditions was approximately 9.5.

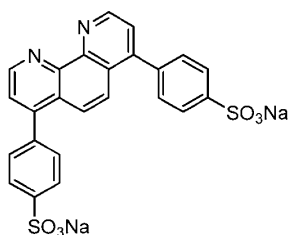
[0171] As shown in **FIG. 3**, TCEP with 50% Pd achieved above 90% cleavage by 5 minutes and close to 100% cleavage at 10 minutes. UCM with 100% Pd was able to achieve about 100% cleavage at 5 minutes. UCM with 50% Pd reached about 60% cleavage at 10 minutes, and reached about 90% cleavage after 60 minutes. The results indicate that TCEP may be suitable for inclusion in reagents where minimization of Pd is desirable.

Example 4. Evaluation of Pd/TCEP/ NH_3BH_3 in Sequencing by Synthesis

[0172] The efficiency of TCEP in the presence of a boron-containing reducing agent NH_3BH_3 in the Pd cleavage mixture were tested on Illumina's iSeq™ against the standard UCM containing Pd/THPP (without a reducing agent). The substrate used for cleavage was a fully functionalized A nucleotide with 3' AOM blocking group and the pH of all conditions was approximately 9.5. As shown in **FIG. 4**, % phasing and % prephasing for the sequencing run with the Pd/TCEP/ NH_3BH_3 cleavage mix were comparable to the standard Pd cleavage mixture.

Example 5. Efficiency of Bidentate N,N non-phosphine ligand

[0173] In this example, a N,N-bidentate non-phosphine ligand having structure



was tested to assess its ability to form Pd(0) from a Pd pre-catalyst, Na_2PdCl_4 (with a 1:2 ligand to Pd ratio) and its ability to cleave an AOM substrate with the presence of an external reducing agent, NH_3BH_3 . As shown in **FIG. 5A**, the N,N-bidentate ligand was capable of forming Pd(0). The standard UCM containing Pd/THPP (without a reducing agent) was used as a control., resulting in 80,551 RFU, while the Pd(0) active catalyst generated from the N,N-bidentate non-phosphine ligand resulted in 42,059 RFU.

[0174] Additionally, the cleavage efficiency of the Pd catalyst generated from the N,N-bidentate non-phosphine ligand was tested. The substrate used for cleavage was a fully functionalized A nucleotide with 3' AOM blocking group. **FIG. 5B** illustrates the percentage cleavage of the 3' AOM blocked substrate after exposure to UCM or a cleavage mixture containing N,N-bidentate non-phosphine ligand at 1 minute and 60 minutes respectively. As shown, the N,N-bidentate non-phosphine ligand cleaved about 14% of the AOM substrate after 1 minute and about 33% of the AOM substrate at 60 minutes, while the UCM cleaved about 49.3% of the AOM substrate at 1 minute and about 98.2% of the AOM substrate at 60 minutes. This experiment demonstrated the usability of N,N-bidentate non-phosphine ligand to form an active Pd(0) catalyst in a chemical cleavage reaction.

WHAT IS CLAIMED IS:

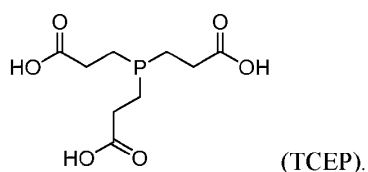
1. A method for determining sequences of a plurality of target polynucleotides, comprising:
 - (a) contacting a solid support with sequencing primers under hybridization conditions, wherein the solid support comprises a plurality of target polynucleotides immobilized thereon; and the sequencing primers are complementary to at least a portion of the target polynucleotides;
 - (b) contacting the solid support with a first aqueous solution comprising DNA polymerase and one or more of four different types of nucleotides under conditions suitable for DNA polymerase-mediated primer extension, wherein each of the nucleotides comprises a 3' blocking group comprising an unsubstituted or substituted allyl group;
 - (c) incorporating one type of nucleotides into the sequencing primers to produce extended copy polynucleotides;
 - (d) performing one or more fluorescent measurements of the extended copy polynucleotides;
 - (e) removing the 3' blocking group of the incorporated nucleotides in a second aqueous solution comprising a transition metal catalyst and one or more reducing agents, the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand, and wherein the one or more reducing agents comprise a boron-containing reducing agent; and
 - (f) washing the solid support with a third aqueous solution after the removal of the 3' blocking group of the incorporated nucleotides.
2. The method of claim 1, further comprising: repeating steps (b) through (f) until sequences of at least a portion of the target polynucleotides are determined.
3. The method of claim 2, wherein steps (b) through (f) are repeated at least 50 times, at least 100 times, at least 150 times, at least 200 times, at least 250 times, or at least 300 times.
4. The method of any one of claims 1 to 3, wherein the transition metal catalyst is Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the water-soluble non-reducing phosphine.
5. The method of any one of claims 1 to 4, wherein the water soluble non-reducing phosphine comprises tris(substituted C₁-C₆ alkyl)phosphine or tris(substituted C₆-C₁₀ aryl)phosphine, or a combination thereof.
6. The method of claim 5, wherein the transition metal catalyst is a Pd(0) catalyst generated *in situ* from the palladium complex and the water soluble non-reducing tris(substituted C₁-C₆ alkyl)phosphine.

7. The method of claim 6, wherein the palladium complex comprises $[\text{Pd}(\text{Allyl})\text{Cl}]_2$, Na_2PdCl_4 , K_2PdCl_4 , Li_2PdCl_4 , $[\text{Pd}(\text{Allyl})(\text{THPP})]\text{Cl}$, $[\text{Pd}(\text{Allyl})(\text{THPP})_2]\text{Cl}$, $\text{Pd}(\text{CH}_3\text{CN})_2\text{Cl}_2$, $\text{Pd}(\text{OAc})_2$, $\text{Pd}(\text{PPh}_3)_4$, $\text{Pd}(\text{dba})_2$, $\text{Pd}(\text{Acac})_2$, $\text{PdCl}_2(\text{COD})$, $\text{Pd}(\text{TFA})_2$, Na_2PdBr_4 , K_2PdBr_4 , PdCl_2 , PdBr_2 , or $\text{Pd}(\text{NO}_3)_2$, or combinations thereof.

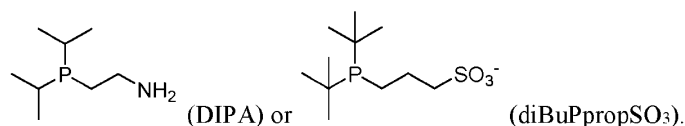
8. The method of claim 7, wherein the palladium complex comprises $[\text{Pd}(\text{Allyl})\text{Cl}]_2$ or Na_2PdCl_4 .

9. The method of any one of claims 5 to 8, wherein the tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphine is a tris(carboxy substituted $\text{C}_1\text{-C}_6$ alkyl)phosphine.

10. The method of claim 9, wherein the tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphine is



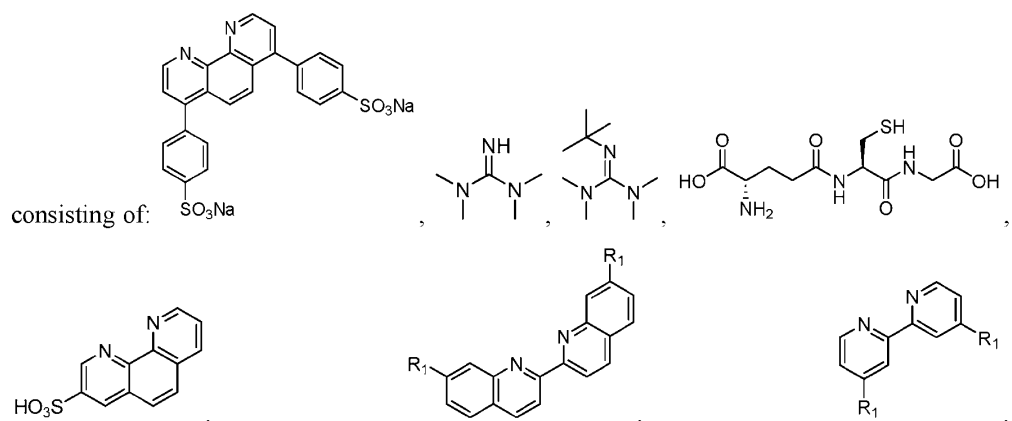
11. The method of any one of claims 5 to 8, wherein the tris(substituted $\text{C}_1\text{-C}_6$ alkyl)phosphine is:



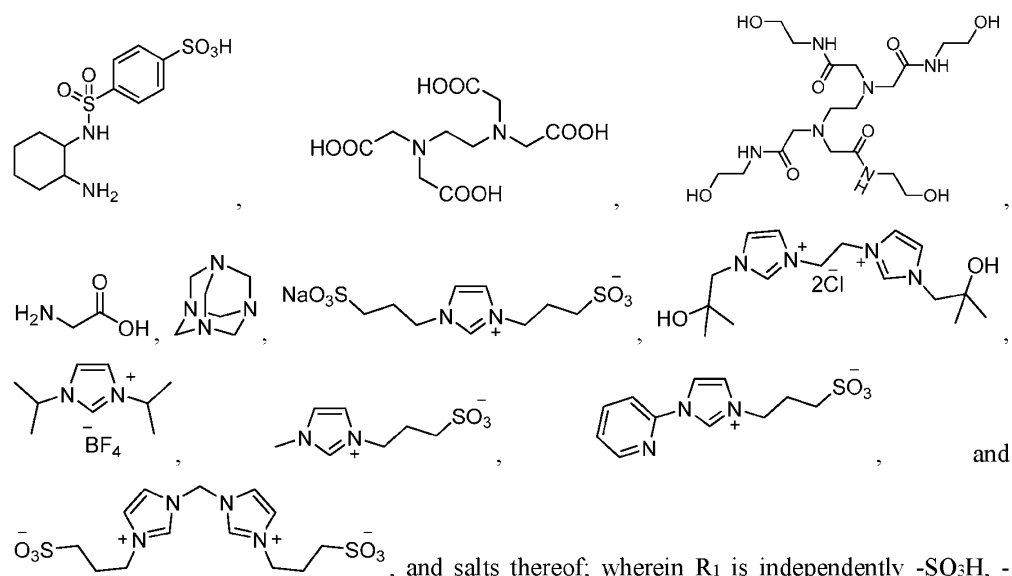
12. The method of any one of claims 1 to 11, wherein the molar ratio of the palladium complex and the water soluble non-reducing phosphine is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10.

13. The method of any one of claims 1 to 3, wherein the transition metal catalyst is Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the N,N-bidentate non-phosphine ligand.

14. The method of claim 13, wherein the N,N-bidentate ligand is selected from the group





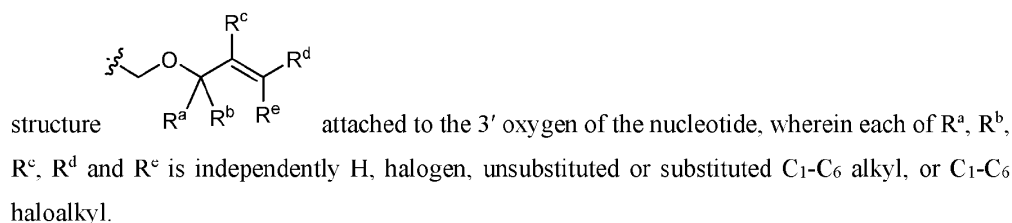


COOH, $-\text{PO}_3\text{H}_2$, or $-\text{NMe}_3^+$; R_2 is $-(\text{CH}_2)_{1-3}\text{COOH}$; and R_3 is $-\text{SO}_3\text{H}$ or $-\text{COOH}$.

15. The method of any one of claims 1 to 14, wherein the boron-containing reducing agent is NH_3BH_3 or $\text{B}_2(\text{OH})_4$.

16. The method of any one of claims 1 to 15, wherein the pH of the second aqueous solution is from about 7.0 to about 10, or from about 7.5 to about 9.5.

17. The method of any one of claims 1 to 16, wherein the 3' blocking group has the



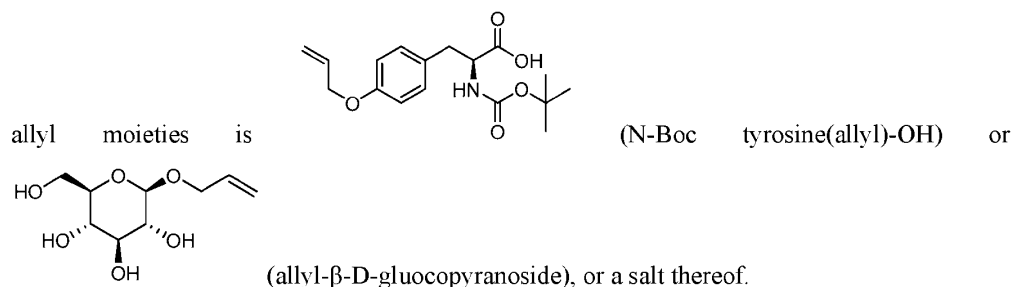
18. The method of claim 17, wherein the 3' blocking group of the nucleotide has the structure  attached to the 3' oxygen of the nucleotide.

19. The method of any one of claims 1 to 18, wherein the first aqueous solution or the third aqueous solution comprises at least one Pd(0) scavenger.

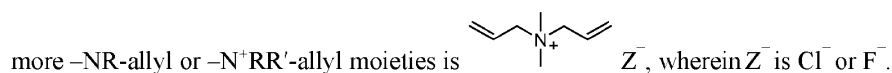
20. The method of claim 19, wherein the Pd(0) scavenger comprises one or more allyl moieties selected from the group consisting of -O-allyl, -S-allyl, -NR-allyl, and -N⁺RR'-allyl, and combinations thereof, wherein R is H, unsubstituted or substituted C₁-C₆ alkyl, unsubstituted or substituted C₂-C₆ alkenyl, unsubstituted or substituted C₂-C₆ alkynyl, unsubstituted or substituted C₆-C₁₀ aryl, unsubstituted or substituted 5 to 10 membered heteroaryl, unsubstituted

or substituted C₃-C₁₀ carbocyclyl, or unsubstituted or substituted 5 to 10 membered heterocyclyl; and R' is H, unsubstituted C₁-C₆ alkyl or substituted C₁-C₆ alkyl.

21. The method of claim 20, wherein the Pd(0) scavenger comprising one or more -O-



22. The method of claim 20, wherein the Pd(0) scavenger comprising one or more one or



23. The method of any one of claims 19 to 22, wherein the Pd(0) scavenger comprising one or more allyl moieties is in the first aqueous solution.

24. The method of any one of claims 1 to 23, wherein the third aqueous solution further comprises at least one Pd(II) scavenger.

25. The method of claim 24, wherein the Pd(II) scavenger comprises L-cysteine or sodium thiosulfate.

26. The method of any one of claims 1 to 25, wherein the 3' blocking group of the incorporated nucleotides is removed in less than 5 minutes when step (e) is performed at room temperature.

27. The method of claim 26, wherein the 3' blocking group of the incorporated nucleotides is removed in about 1 minute.

28. The method of any one of claims 1 to 27, wherein the solid support comprises an array of immobilized target polynucleotides.

29. A kit for use with a sequencing apparatus, comprising:

an aqueous cleavage mixture comprising a transition metal catalyst and one or more reducing agents, wherein the transition metal catalyst is generated from a palladium or nickel complex with a water-soluble non-reducing phosphine or a N,N-bidentate non-phosphine ligand, and wherein the one or more reducing agents comprise a boron-containing reducing agent.

30. The kit of claim 29, wherein the transition metal catalyst is a Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the water-soluble non-reducing phosphine.

31. The kit of claim 29 or 30, wherein the water soluble non-reducing phosphine comprises tris(substituted C₁-C₆ alkyl)phosphine or tris(substituted C₆-C₁₀ aryl)phosphine, or a combination thereof.

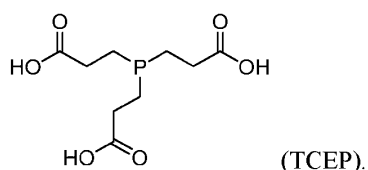
32. The kit of claim 31, wherein the transition metal catalyst is a Pd(0) catalyst generated *in situ* from the palladium complex and the water-soluble non-reducing tris(substituted C₁-C₆ alkyl)phosphine.

33. The kit of claim 32 wherein the palladium complex comprises [Pd(Allyl)Cl]₂, Na₂PdCl₄, K₂PdCl₄, Li₂PdCl₄, [Pd(Allyl)(THPP)]Cl, [Pd(Allyl)(THPP)]₂Cl, Pd(CH₃CN)₂Cl₂, Pd(OAc)₂, Pd(PPh₃)₄, Pd(dba)₂, Pd(Acac)₂, PdCl₂(COD), Pd(TFA)₂, Na₂PdBr₄, K₂PdBr₄, PdCl₂, PdBr₂, or Pd(NO₃)₂, or combinations thereof.

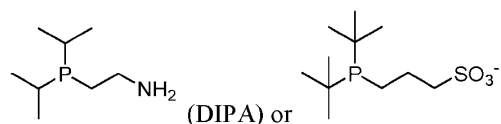
34. The kit of claim 33, wherein the palladium complex comprises [Pd(Allyl)Cl]₂ or Na₂PdCl₄.

35. The kit of any one of claims 31 to 34, wherein the tris(substituted C₁-C₆ alkyl)phosphine is a tris(carboxy substituted C₁-C₆ alkyl)phosphine.

36. The kit of claim 35, wherein the tris(substituted C₁-C₆ alkyl)phosphine is



37. The kit of any one of claims 31 to 34, wherein the tris(substituted C₁-C₆ alkyl)phosphine is:



38. The kit of any one of claims 29 to 37, wherein the molar ratio of the palladium complex and the water soluble non-reducing phosphine is about 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5 or 1:10.

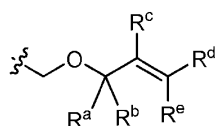
39. The kit of claim 29, wherein the transition metal catalyst is a Pd(0) catalyst, and the Pd(0) catalyst is generated from the palladium complex with the N,N-bidentate non-phosphine ligand.

40. The kit of any one of claims 29 to 39, wherein the boron-containing reducing agent is NH₃BH₃ or B₂(OH)₄.

41. The kit of any one of claims 29 to 40, wherein the pH of the aqueous cleavage mixture is from about 7.0 to about 10, or from about 7.5 to about 9.5.

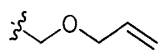
42. The kit of any one of claims 29 to 41, further comprising an incorporation mixture, wherein the incorporation mixture comprises one or more of four different types of nucleotides, wherein each of the nucleotides has a 3' blocking group comprising an unsubstituted or substituted allyl group, and at least one Pd(0) scavenger.

43. The kit of claim 42, wherein the 3' blocking group of the nucleotide has the structure



attached to the 3' oxygen of the nucleotide, wherein each of R^a , R^b , R^c , R^d and R^e is independently H, halogen, unsubstituted or substituted C_1 - C_6 alkyl, or C_1 - C_6 haloalkyl.

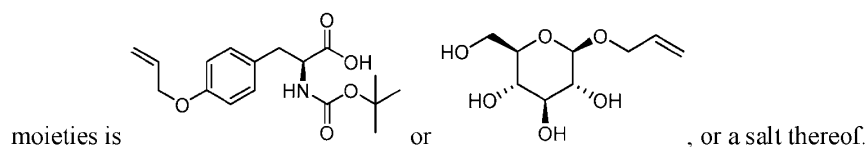
44. The kit of claim 43, wherein the 3' blocking group of the nucleotide has the structure



attached to the 3' oxygen of the nucleotide.

45. The kit of any one of claims 42 to 44, wherein the Pd(0) scavenger comprises one or more allyl moieties selected from the group consisting of $-O$ -allyl, $-S$ -allyl, $-NR$ -allyl, and $-N^+RR'$ -allyl, and combinations thereof, wherein R is H, unsubstituted or substituted C_1 - C_6 alkyl, unsubstituted or substituted C_2 - C_6 alkenyl, unsubstituted or substituted C_2 - C_6 alkynyl, unsubstituted or substituted C_6 - C_{10} aryl, unsubstituted or substituted 5 to 10 membered heteroaryl, unsubstituted or substituted C_3 - C_{10} carbocyclyl, or unsubstituted or substituted 5 to 10 membered heterocyclyl; and R' is H, unsubstituted C_1 - C_6 alkyl or substituted C_1 - C_6 alkyl.

46. The kit of claim 45, wherein the Pd(0) scavenger comprising one or more $-O$ -allyl

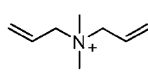


moieties is

or

, or a salt thereof.

47. The kit of claim 45, wherein the Pd(0) scavenger comprising one or more one or more

$-NR$ -allyl or $-N^+RR'$ -allyl moieties is  Z^- , wherein Z^- is Cl^- or F^- .

48. The kit of any one of claims 42 to 47, wherein the incorporation mixture is in a lyophilized form.

49. The kit of any one of claims 29 to 48, further comprising an aqueous wash solution, or a composition that is reconstitutable into an aqueous wash solution.

50. The kit of claim 49, wherein the aqueous wash solution comprises at least one Pd(II) scavenger.

51. A cartridge for use with a sequencing apparatus, comprising a plurality of chambers, wherein one of the plurality of chambers is for use with the kit comprising the aqueous cleavage mixture according to any one of claims 29 to 41.