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(54) Titre : PROCEDE ET SYSTEME DE SEQUENCAGE D'ACIDES NUCLEIQUES
(54) Title: METHOD AND SYSTEM FOR SEQUENCING NUCLEIC ACIDS

(57) Abrégé/Abstract:
Provided are compositions, methods and systems for determining the sequence of a template nucleic acid using a polymerase-based, sequencing-by-binding procedure. An examination step involves monitoring the interaction between a polymerase and template nucleic acid in the presence of one or more nucleotides. Identity of the next correct nucleotide in the sequence is determined without incorporation of any nucleotide into the structure of the primer by formation of a phosphodiester bond. An optional incorporation step can be used after the examination step to extend the primer by one or more nucleotides, thereby incrementing the template nucleotides that can be examined in a subsequent examination step. The sequencing-by-binding procedure does not require the use of labeled nucleotides or polymerases, but optionally can employ these reagents.

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**(54) Title:** METHOD AND SYSTEM FOR SEQUENCING NUCLEIC ACIDS

**(57) Abstract:** Provided are compositions, methods and systems for determining the sequence of a template nucleic acid using a polymerase-based, sequencing-by-binding procedure. An examination step involves monitoring the interaction between a polymerase and template nucleic acid in the presence of one or more nucleotides. Identity of the next correct nucleotide in the sequence is determined without incorporation of any nucleotide into the structure of the primer by formation of a phosphodiester bond. An optional incorporation step can be used after the examination step to extend the primer by one or more nucleotides, thereby incrementing the template nucleotides that can be examined in a subsequent examination step. The sequencing-by-binding procedure does not require the use of labeled nucleotides or polymerases, but optionally can employ these reagents.

# METHOD AND SYSTEM FOR SEQUENCING NUCLEIC ACIDS

## Related Applications

**[0001]** This application claims the benefit of U.S. Provisional Application No. 62/447,319, filed January 17, 2017; and U.S. Provisional Application No. 62/375,379, filed August 15, 2016. The disclosures of these earlier applications are hereby incorporated by reference in their entireties.

## Technical Field

**[0002]** The present disclosure relates generally to the field of biotechnology. More particularly, the disclosure concerns nucleic acid sequencing technology. Still more particularly, the disclosure concerns sequencing-by-binding techniques that identify a next correct nucleotide independent of nucleotide incorporation.

## Background

**[0003]** The determination of nucleic acid sequence information is now an important part of biological and medical research. For example, nucleic acid sequence information is helpful for identifying genes associated with certain diseases and phenotypes, identifying potential drug targets, and understanding the mechanisms of disease development and progress. Sequence information also is an important part of personalized medicine, where it can be used to optimize the diagnosis, treatment, or prevention of disease in a specific subject.

**[0004]** High-throughput, cost-effective nucleic acid sequencing has the potential to usher in a new era of research and personalized medicine. Several commercial sequencing platforms are available, but remain prohibitively expensive for genetic analysis in the mass-market.

**[0005]** Currently, a variety of sequencing technologies utilize a method known alternatively as "sequencing-by-synthesis" (SBS) or "sequencing by incorporation." This method commonly employs a polymerase to synthesize a DNA strand complementary to a template strand that is to be sequenced. This may involve providing nucleotides or short oligonucleotides, which are modified with identifying tags, so that the base type of the incorporated nucleotide or oligonucleotide is detected as synthesis proceeds. Detection may be in real-time, where the nucleotides are detected as they are incorporated. Unfortunately, real-time procedures can sometimes suffer from inaccurate reads of regions containing highly repetitive sequences and homopolymeric stretches. Detection may also proceed in iterations

of stop and proceed steps, wherein controlled reaction conditions and/or reagents reversibly stop and start the reaction at a given time during synthesis.

**[0006]** As many sequencing-by-synthesis technologies are based on fluorescent detection, fluorescent labeling of nucleotides is required. The necessary illumination and optical systems can increase complexity and expense of the system. By way of example, SBS methods often require fluorescently labeled dNTPs for detecting incorporated nucleotides and identifying a template nucleic acid sequence. However, the use of labeled nucleotides has limitations on accuracy, since current SBS reactions using labeled nucleotides become error-prone after a few hundred bases. Even a 1% error rate could compromise the significance of the sequencing results when an entire genome is to be analyzed. Accuracy may be decreased when a failure to detect a single label results in a deletion error or when the detection of a stray molecule results in an insertion error. Fluorophores which are bleached cause false-negatives. In addition, contamination of labeled dNTPs by unlabeled dNTPs (*e.g.*, impurities or hydrolysis products) can also cause false-negatives. Still further, stray signals from labeled dNTPs non-specifically bound to a structured surface contribute to insertion errors or high signal to noise ratios. The use of modified nucleotides significantly slows enzyme kinetics, thereby making the sequencing reaction very slow. Another challenge with labeled nucleotides in SBS procedures is that the label needs to be removed or deactivated after it is incorporated and detected, so that the next addition can be observed without background signal. Thus, to obtain long read-lengths, each addition must be followed by virtually 100% chemical, enzymatic or photolytic steps to unblock the substrate or remove the dye for the next addition.

**[0007]** Disclosed below is a technical approach that overcomes many of the problems typically associated with prior sequencing technologies.

## Brief Description of the Drawings

**[0008]** Figure 1 is a graph showing the results of an experiment using non-labeled optical detection methods where magnesium was present or absent during the binding or examination step.

**[0009]** Figure 2 is a graph showing sequencing using Bst enzyme binding kinetics for determining the correct base using Bst2.0 enzyme and dNTPs.

**[0010]** Figure 3 is a graph showing the effects of salt concentration on match and mismatch base discrimination effects using biolayer interferometry on a FORTEBIO® octet instrument (Menlo Park, CA).

**[0011]** Figure 4 is a graph showing base discrimination during the wash step, i.e., during dissociation of the polymerase, using phiX_matchC and FP2 primer and klenow or Bst2.0 enzyme, and SrCl2.

**[0012]** Figure 5 is a graph showing the effect of washing on the stabilization of nucleic acid, polymerase complex using varying concentrations of SrCl2 (0 mM-14 mM).

**[0013]** Figure 6 is a graph showing the effect of 3'-5' exonuclease activity of DNA pol I on sequencing.

**[0014]** Figures 7A and 7B are graphs showing sequencing of human ALK gatekeeper region using HIV-1 reverse transcriptase, NNRTI compound 7 and the indicated dNTPs. Figure 7A is a graph showing the time course for consecutive cycles of sequencing. Figure 7B is a graph showing cycles 1-12 in individual panels after subtracting background from the previous cycle. The expected sequence read was CAGCAGGA (SEQ ID NO:1) and the observed sequence read was CAGCAGG (SEQ ID NO:2).

**[0015]** Figure 8 is a graph showing sequencing of human ALK gatekeeper region using HIV-1 reverse transcriptase, NNRTI compound 18 and the various dNTPs.

**[0016]** Figure 9 is a sensorgram showing sequencing of the phiX_matchA template using an SPRi biosensor. Grayed areas correspond to correct base calls. The dotted line indicates the intensity change threshold used to determine binding of the correct Klenow/dNTP combination.

**[0017]** Figures 10A, 10B, and 10C are graphs showing sequencing of dsDNA by nick translation using Bst DNA Polymerase from Bacillus stearothermophilus. Double-stranded DNA with one base gap was treated with Bst DNA Pol with or without the indicated dNTP in Binding Buffer. Biosensors were transferred to Reaction Buffer for dNTP incorporation followed by transfer to Reaction Buffer containing Bst DNA Pol without dNTP for 5'-3' exonucleolytic cleavage of the non-template strand for 120 seconds (Figure 10A) or 60 seconds (Figure 10B). As a control, Bst DNA Pol was used for sequencing-by-binding a primed ssDNA template, dNTP incorporation followed by 5'-3' exonucleolytic processing for 60 seconds (Figure 10C).

**[0018]** Figures 11A, 11B, and 11C are graphs showing sequencing of dsDNA with 5'-flap by strand displacement using Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase. DNA templates were treated with Klenow exo(-) DNA Pol with or without the indicated dNTP in Binding Buffer without MgCl2. Biosensors were transferred to Wash Buffer with MgCl2 for catalysis followed by re-equilibration in Binding Buffer without enzyme or dNTP. Cycles were repeated for each individual dNTP as indicated. Figure 11A: Single-stranded

DNA. Figure 11B: double-stranded DNA with one base gap. Figure 11C: double-stranded DNA with a 5'-oligo-dT flap downstream of a one base pair gap.

**[0019]** Figures 12A, 12B, and 12C are graphs showing sequencing ssDNA by Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase are promoted by salt components. Binding Buffers contain 200 mM glutamate (Figure 12A), 100 mM glutamate (Figure 12B) and 50 mM glutamate (Figure 12C). Reaction Buffers contain MgCl2 without glutamate. The applied dNTP for each cycle ("dNTP") is shown in the top text row (SEQ ID NO:14) of each of Figures 12A, 12B, and 12C. Binding of Klenow (exo(-)) indicates observed sequence ("Observed") in the second row of Figures 12A (SEQ ID NO:15), 12B (SEQ ID NO:17), and 12C (SEQ ID NO:19). "Expected" sequence based on the template is shown in the third text row of Figures 12A (SEQ ID NO:16), 12B (SEQ ID NO:18), and 12C (SEQ ID NO:20).

**[0020]** Figures 13A, 13B, and 13C are graphs of sequencing the sense strand of human ALK C4493A mutant in a background of ALK wild-type by Klenow exo(-) DNA polymerase. Figure 13 A is a sensorgram demonstrating sequencing in ssDNA mixtures of wild-type and C4493A mutant. Figure 13B is a graph showing, in ssDNA mixtures, linear quantitation of C4493A mutant shown in Cycle 4 (T), and linear quantitation of wild-type ALK shown in cycle 3 (G). Figure 13C is a graph showing, in dsDNA-flap mixtures, linear quantitation of C4493A mutant is shown in Cycle 4 (T), and roughly linear quantities of wild-type ALK are shown in cycle 3 (G).

**[0021]** Figures 14A and 14B are graphs of divalent cation-mediated binding of Klenow exo(-) and dCTP to human ALK C4493A mutant and dissociation with or without catalytic metal (MgCl2). Figure 14A is a graph showing binding to the primer/template and dissociation in the presence of non-catalytic metals. Figure 14B is a graph showing binding to the primer/template and dissociation in the presence of catalytic metals.

**[0022]** Figure 15 is a graph of Klenow exo(-) sequencing of human ALK C4493A mutant in which binding is mediated by low concentration of CoCl2, and incorporation is mediated by high concentration of CoCl2.

**[0023]** Figure 16 is a graph of Klenow exo(-) sequencing of human ALK C4493A mutant in which binding is mediated by Ni(II)SO4, and incorporation is effected by MgCl2. Dissociation to baseline is observed only in the presence of nucleoside diphosphate kinase and ADP, which scavenge free dNTP thus prevent re-binding of dNTP into the complex of Klenow exo(-) and primer/template.

**[0024]** Figures 17A and 17B are graphs showing homopolymer resolution during Bsu Pol I (large fragment) sequencing of human ALK C4493A mutant. Binding is mediated by

Ni(II)SO4, incorporation by MgCl2, and dissociation in the presence or absence of dNDP. Figure 17A is a sensorgram of sequencing using the Octet QK system. Figure 17B is a graph showing resolution of two consecutive G peaks is dependent upon the concentrations of dNDP in the Reaction Buffer.

**[0025]** Figures 18A, 18B, 18C, 18D and 18E are graphs showing homopolymer resolution during Bsu Pol I (large fragment) sequencing of human wild-type ALK. Figure 18A is a sensogram of Ni enhanced binding of polymerase on templates ALK-G1, ALK-G2 and ALK-G3. Figure 18B is a sensogram of Ni enhanced binding of polymerase on template ALK-G4. Figure 18C is a sensogram of the incorporation/dissociation time following addition of reaction buffer containing Ni and Mg. Figure 18D is a graph showing examination phase parameters plotted versus the number of G nucleotides in the primer strand needed to fill the C homopolymer of the template. Control is a single G incorporation (G), and "**" indicates statistically significant results with $p<0.01$. Figure 18E is a graph showing initial rates observed during the incorporation/dissociation phase plotted for indicated Bsu polymerase concentrations. Control is a single G incorporation (G).

**[0026]** Figure 19 is a graphical interferometry trace showing the magnitude of polymerase binding (vertical axis) as a function of reaction progress (horizontal axis). Traces represent binding to four different biosensor tips using different concentrations of non-immobilized primed template nucleic acid in the polymerase delivery reagent (PDR). Signals measured at the end of the period of binary complex formation progressed lower as the non-immobilized primed template nucleic acid molecule in the PDR increased in concentration (i.e., the traces corresponding to 0 nM, 10 nM, 100 nM, and 1,000 nM concentrations).

**[0027]** Figure 20 is a bar graph plotting differences in magnitude of binary and ternary complex signals achieved using the indicated concentrations of non-immobilized primed template nucleic acid in the PDR. The PDR including 100 nM of the non-immobilized primed template nucleic acid molecule gave the most dramatic difference in signal magnitude.

**[0028]** Figure 21 is a workflow diagram illustrating a sequencing-by-binding protocol employing detectably labeled polymerase.

**[0029]** Figure 22 is a bar graph presenting fluorescence intensity (vertical axis) as a function of cycle number (horizontal axis), where each cycle involved binding of labeled polymerase and one nucleotide at a time.

## Summary

**[0030]** Provided herein are methods for determining the sequence of a template nucleic acid molecule, where the methods are based on a binding reaction carried out under specified conditions. The method generally includes an examination step prior to incorporation of a nucleotide. The examination step involves providing a template nucleic acid molecule primed with a primer; contacting the primed template nucleic acid molecule with a first reaction mixture that includes a polymerase and at least one nucleotide molecule; monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of the nucleotide molecule, without chemical incorporation of the nucleotide molecule into the primed template nucleic acid; and identifying a next base in the template nucleic acid using the monitored interaction of the polymerase with the primed template nucleic acid molecule in the presence of the nucleotide molecule. In this procedure, ternary complex stabilization and binary complex destabilization advantageously enhance discrimination between correct and incorrect nucleotides.

**[0031]** Also provided herein is a method of determining the identity of the next correct nucleotide for a primed template nucleic acid molecule. The method can include steps of (a) providing a template nucleic acid molecule primed with a primer; (b) serially contacting the primed template nucleic acid molecule with reaction mixtures each including a polymerase and a different combination of two or three different test nucleotides under conditions that stabilize ternary complexes including the primed template nucleic acid molecule, the polymerase and a next correct nucleotide, while precluding incorporation of any nucleotide into the primer; (c) detecting interaction of the polymerase with the primed template nucleic acid molecule without chemical incorporation of any nucleotide into the primer of the primed template nucleic acid molecule, to determine whether ternary complexes form; and (d) determining whether a test nucleotide that is common to at least two of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction. Optionally, the method further includes (e) incorporating a nucleotide at the 3' end of the primer after step (c). In a further option, the nucleotide that is incorporated is an unlabeled nucleotide or an unlabeled reversible terminator nucleotide. Optionally, steps (b) through (e) can be repeated to sequence the primed template nucleic acid molecule.

**[0032]** In some embodiments, step (d) of the above method includes determining whether a test nucleotide that is common to two of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction. In this embodiment, four different reaction mixtures can be serially contacted with the primed

template nucleic acid molecule in step (b), wherein in aggregate each different nucleotide is present in two reaction mixtures.

**[0033]** In other embodiments, step (d) of the above method includes determining whether a test nucleotide that is common to three of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction. In this embodiment, six different reaction mixtures can be serially contacted with the primed template nucleic acid molecule in step (b), wherein in aggregate each different nucleotide is present in three reaction mixtures.

**[0034]** In particular embodiments, the conditions of step (b) also destabilize binary complexes that include the primed template nucleic acid molecule and the polymerase but not the next correct nucleotide.

**[0035]** The ternary complex of step (b) can be stabilized by the presence of a reversible terminator moiety on the 3' terminal nucleotide of the primer. Optionally, after step (c), the method can include a step of removing the reversible terminator moiety on the 3' terminal nucleotide of the primer. In some embodiments of the above method of determining the identity of the next correct nucleotide for a primed template nucleic acid molecule, each of the test nucleotides is an unlabeled nucleotide. Optionally, the polymerase includes an exogenous label that is detected in step (c). Alternatively or additionally, the next correct nucleotide includes an exogenous label that is detected in step (c).

**[0036]** Further provided is a method of sequencing a primed template nucleic acid. The method can include steps of (a) contacting a primed template nucleic acid with a polymerase and a first combination of two or three types of test nucleotides under conditions that form a stabilized ternary complex between the polymerase, primed template nucleic acid and a test nucleotide that is complementary to the next base of the primed template nucleic acid; (b) detecting the ternary complex while precluding incorporation of test nucleotides into the primer; (c) repeating steps (a) and (b) using the primed template nucleic acid, a polymerase and a second combination of two or three types of test nucleotides, wherein the second combination is different from the first combination; (d) incorporating into the primer, after step (c), a nucleotide that is complimentary to the next base; and (e) repeating steps (a) through (d) to identify a sequence of the primed template nucleic acid.

**[0037]** In some embodiments of the above sequencing method, the first combination includes two, and only two, types of test nucleotides. Optionally, the second combination can also include two, and only two, types of test nucleotides.

**[0038]** In some embodiments of the above sequencing method, steps (a) and (b) are carried out serially for four different combinations of two types of test nucleotides, wherein each different nucleotide type is contacted with the primed template nucleic acid two times in aggregate. Alternatively, steps (a) and (b) can be carried out serially for six different combinations of two types of test nucleotides, wherein each different nucleotide type is present three times in aggregate.

**[0039]** Further provided is a method of determining the identity of the next correct nucleotide for a primed template nucleic acid molecule. The method includes the steps of: (a) providing a template nucleic acid molecule primed with a primer; (b) contacting the primed template nucleic acid molecule from step (a) with a first reaction mixture including a polymerase and at least one test nucleotide under conditions that (i) stabilize ternary complexes including the primed template nucleic acid molecule, the polymerase and a next correct nucleotide, while precluding incorporation of any nucleotide into the primer, and (ii) destabilize binary complexes including the primed template nucleic acid molecule and the polymerase but not the next correct nucleotide; (c) detecting (e.g., monitoring) interaction of the polymerase with the primed template nucleic acid molecule without chemical incorporation of any nucleotide into the primer of the primed template nucleic acid molecule, to determine whether a ternary complex formed in step (b); and (d) determining whether any of the test nucleotides is the next correct nucleotide for the primed template nucleic acid molecule using the result of step (c). According to one generally preferred embodiment, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by including in the first reaction mixture a non-catalytic metal ion that inhibits polymerization. According to another generally preferred embodiment, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by including in the first reaction mixture a polymerase inhibitor selected from the group consisting of an allosteric polymerase inhibitor, an uncompetitive polymerase inhibitor, a competitive polymerase inhibitor, and a non-competitive polymerase inhibitor. According to another generally preferred embodiment, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by terminating the primer with a reversible terminator nucleotide before conducting step (b). According to another generally preferred embodiment, the conditions that destabilize binary complexes in step (b) can include a concentration of from 50 mM to 1,500 mM of a salt that provides monovalent cations, the salt being included in the first reaction mixture. More preferably, the reaction conditions that stabilize ternary complexes and destabilize binary

complexes in step (b) can enhance ternary complex formation over binary complex formation by at least two-fold. Still more preferably, the reaction conditions that stabilize ternary complexes and destabilize binary complexes in step (b) can enhance ternary complex formation over binary complex formation by at least five-fold. Alternatively, when step (b) includes a concentration of from 50 mM to 1,500 mM of the salt that provides monovalent cations in the first reaction mixture, the salt that provides monovalent cations can further provide glutamate anions. Alternatively, when step (b) includes a concentration of from 50 mM to 1,500 mM of the salt that provides monovalent cations in the first reaction mixture, the concentration of the salt that provides monovalent cations can be from 50 mM to 500 mM. More preferably, the concentration of the salt that provides monovalent cations can be from 100 mM to 300 mM. Alternatively, when step (b) includes a concentration of from 50 mM to 500 mM of the salt that provides monovalent cations in the first reaction mixture, the first reaction mixture can further include a glutamate salt at a concentration of from 10 mM to 1.6 M. When this is the case, the concentration of the glutamate salt can be from 80 mM to 320 mM. Alternatively, when step (b) includes a concentration of from 50 mM to 500 mM of the salt that provides monovalent cations in the first reaction mixture, the salt can be a glutamate salt. Alternatively, when step (b) includes a concentration of from 50 mM to 500 mM of the salt that provides monovalent cations in the first reaction mixture, the salt can be selected from the group consisting of NaCl, KCl, NH2(SO4), and potassium glutamate. According to another embodiment, when step (b) includes a concentration of from 50 mM to 1,500 mM of the salt that provides monovalent cations in the first reaction mixture, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by a non-catalytic metal ion that inhibits polymerization. According to another embodiment, when step (b) includes a concentration of from 50 mM to 1,500 mM of the salt that provides monovalent cations in the first reaction mixture, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by terminating the primer with a reversible terminator nucleotide before performing step (b). According to another embodiment, when step (b) includes a concentration of from 50 mM to 500 mM of the salt that provides monovalent cations in the first reaction mixture, the conditions that destabilize binary complexes in step (b) can include 200 mM of the salt that provides monovalent cations. More preferably, the salt can be selected from the group consisting of NaCl, KCl, NH2(SO4), and potassium glutamate. Alternatively, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by a non-catalytic metal ion

that inhibits polymerization. More preferably, the first reaction mixture can include potassium glutamate at a concentration between 10 mM and 1.6 M. According to another embodiment, when step (b) includes a concentration of from 50 mM to 500 mM of the salt that provides monovalent cations in the first reaction mixture, and when the conditions that destabilize binary complexes in step (b) include 200 mM of the salt that provides monovalent cations, the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer can be provided by terminating the primer with a reversible terminator nucleotide before performing step (b). According to another generally preferred embodiment, each of the test nucleotides of the first reaction mixture is a different unlabeled nucleotide. More preferably, each of the different unlabeled nucleotides can be a different native nucleotide. Alternatively, the polymerase can include an exogenous detectable label. For example, the exogenous detectable label can include a fluorescent label. According to a different preferred embodiment, when each of the test nucleotides of the first reaction mixture is a different unlabeled nucleotide, the polymerase can include a detectable label that emits a signal, and emission of the signal by the detectable label can be substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide. According to another generally preferred embodiment, the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support, the polymerase includes a detectable label that emits a signal, where emission of the signal by the detectable label is substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide, step (c) includes measuring intensity of the signal at the locus on the solid support, and increased formation of the ternary complex in step (b) is indicated by increased intensity of the signal at the locus. When this is the case, the solid support can be contained within a flow cell, and the detectable label can be a fluorescent detectable label. Alternatively, the method can further include the step of (e) replacing the first reaction mixture with a second reaction mixture that includes a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of the one or more nucleotides incorporates into the primer. More preferably, the one or more nucleotides of the second reaction mixture includes at least one reversible terminator nucleotide. Still more preferably, the solid support is contained within a flow cell, and step (e) includes replacing the first reaction mixture by fluid flow through the flow cell. According to another preferred embodiment, when the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support, when the polymerase includes a detectable label that emits a signal, where emission of the signal by the detectable label is

substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide, when step (c) includes measuring intensity of the signal at the locus on the solid support, when increased formation of the ternary complex in step (b) is indicated by increased intensity of the signal at the locus, and when the method further includes the step of (e) replacing the first reaction mixture with a second reaction mixture that includes a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of the one or more nucleotides incorporates into the primer, the second reaction mixture can include less than 100 mM of each of NaCl and KCl. According to another preferred embodiment, when the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support, when the polymerase includes a detectable label that emits a signal, where emission of the signal by the detectable label is substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide, when step (c) includes measuring intensity of the signal at the locus on the solid support, when increased formation of the ternary complex in step (b) is indicated by increased intensity of the signal at the locus, and when the method further includes the step of (e) replacing the first reaction mixture with a second reaction mixture that includes a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of the one or more nucleotides incorporates into the primer, the polymerase of the second reaction mixture can be a different polymerase than the polymerase of the first reaction mixture. In some embodiments, the reversible terminator nucleotide does not include a fluorescent label. According to another generally preferred embodiment, the primed template nucleic acid molecule of step (a) can be immobilized at a locus on a solid support contained within a flow cell, and the method further includes the step of (e) replacing, by fluid flow through the flow cell, the first reaction mixture with a second reaction mixture including a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of the one or more nucleotides incorporates into the primer. According to one preferred embodiment, the one or more nucleotides of the second reaction mixture can include at least one reversible terminator nucleotide. According to a different preferred embodiment, the second reaction mixture includes less than 100 mM of each of NaCl and KCl. According to still a different preferred embodiment, the method further includes a step for washing the immobilized primed template nucleic acid molecule between step (c) and step (e) to remove at least one of the components of the first reaction mixture. According to still yet another preferred embodiment, the polymerase of the second reaction mixture is a different polymerase than the polymerase of the first reaction mixture. In some

embodiments, the reversible terminator nucleotide does not include a fluorescent label. According to another generally preferred embodiment, the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support, step (c) includes measuring intensity of a signal indicating interaction of the polymerase with the primed template nucleic acid molecule at the locus on the solid support, and step (d) includes determining that one of the test nucleotides of the first reaction mixture is the next correct nucleotide for the primed template nucleic acid molecule when the measured intensity of the signal exceeds a predetermined threshold. More preferably, there is the further step of washing the immobilized primed template nucleic acid molecule after step (c) and before step (e) to remove at least one of the components of the first reaction mixture. According to another generally preferred embodiment, the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support contained within a flow cell, and the method further includes the steps of: washing the immobilized primed template nucleic acid molecule after step (c) to remove one or more of the at least one test nucleotide of the first reaction mixture; and detecting (e.g., monitoring) interaction of the polymerase with the immobilized primed template nucleic acid molecule after the washing step to determine whether there remains any of the ternary complex that may have formed in step (b).

**[0040]** Further provided is a method of delivering polymerase and nucleotide to a population of immobilized nucleic acid features, where each feature includes a primed template nucleic acid molecule. The method includes the step of: (a) contacting the population of immobilized nucleic acid features with a first reagent that includes a polymerase, a first nucleotide, and at least one non-immobilized primed template nucleic acid molecule, wherein the first nucleotide is not the next correct nucleotide for any of the non-immobilized primed template nucleic acid molecules of the first reagent, wherein the contacting takes place under conditions that stabilize ternary complexes and inhibit or preclude catalysis of phosphodiester bond formation by the polymerase, and whereby, compared to the use of reagents including the polymerase and the first nucleotide in the absence of the non-immobilized primed template nucleic acid molecule of the first reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule is reduced. In some embodiments, after step (a) there is the further step of (b) replacing the first reagent with a second reagent including the polymerase of the first reagent, a second nucleotide, and at least one non-immobilized primed template nucleic acid molecule, wherein the first and second nucleotides are different from each other, wherein the second nucleotide is not the cognate nucleotide for any of the non-immobilized primed template nucleic acid molecules of the second reagent,

wherein the replacing takes place under conditions that stabilize ternary complexes and inhibit or preclude catalysis of phosphodiester bond formation by the polymerase, and whereby, compared to the use of reagents including the polymerase and the second nucleotide in the absence of the non-immobilized primed template nucleic acid molecule of the second reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule is reduced. In some embodiments, the non-immobilized primed template nucleic acid molecules of the first and second reagents are different from each other.

**[0041]** Further provided is a reaction mixture that includes each of: (a) a plurality of immobilized nucleic acid features, each feature including an immobilized primed template nucleic acid; (b) a polymerase; (c) a non-immobilized primed template nucleic acid molecule; and (d) a nucleotide, wherein the nucleotide is not the cognate nucleotide for the non-immobilized primed template nucleic acid molecule, and wherein nucleotide-independent binding of the polymerase to the immobilized primed template nucleic acid is reduced by the presence of the non-immobilized primed template nucleic acid molecule. In some embodiments, the polymerase, the immobilized primed template nucleic acid, and the nucleotide form a stabilized ternary complex that is inhibited or precluded from incorporating the nucleotide into the immobilized primed template nucleic acid.

**[0042]** Further provided is a polymerase delivery reagent that includes each of: (a) a polymerase; (b) at least one non-immobilized primed template nucleic acid molecule; and (c) at least one nucleotide, wherein none of the nucleotides is a next correct nucleotide for any of the non-immobilized primed template nucleic acid molecules. In some embodiments, the polymerase delivery reagent further includes (d) a ternary complex stabilizing agent that inhibits phosphodiester bond formation by the solution-phase polymerase. In some embodiments, the at least one nucleotide includes at least one native nucleotide. In some embodiments, the solution-phase primed template nucleic acid molecule includes no more than three different types of primed template nucleic acid molecule, each with a different 3' terminal nucleotide. In some embodiments, the polymerase is a mutant polymerase without catalytic phosphodiester bond forming activity in the presence of Mg2+ ions.

**[0043]** Further provided is a method of identifying the next correct nucleotide for a primed template nucleic acid molecule. The method includes the step of (a) providing a template nucleic acid molecule primed with a primer, where the primer includes a reversible terminator moiety attached to its 3' terminal nucleotide. There also can be the step of (b) serially contacting the primed template nucleic acid molecule with a plurality of polymerase-nucleotide combinations in the presence of a catalytic metal ion, and without incorporating

any nucleotide. Each of the combinations can include a polymerase and a different nucleotide. As a consequence of the serial contacting, there is formed a ternary complex including the polymerase, one of the different nucleotides delivered in combination with the polymerase, and the primed template nucleic acid molecule when the one of the different nucleotides is the next correct nucleotide. There also can be the step of (c) detecting the ternary complex, thereby identifying the next correct nucleotide for the primed template nucleic acid molecule as the one of the different nucleotides that contacted the primed template nucleic acid molecule in combination with the polymerase to form the ternary complex. According to one generally preferred embodiment, step (b) can involve serially contacting the primed template nucleic acid molecule with the plurality of polymerase-nucleotide combinations in the presence of the catalytic metal ion and in the absence of any non-catalytic ion that inhibits incorporation. In some embodiments, the catalytic metal ion is magnesium ion. In some embodiments, the polymerase is a labeled polymerase that includes an exogenous detectable label that produces a signal, where the signal produced by the label of the labeled polymerase does not substantially change in the presence or absence of any nucleotide, and step (c) can involve detecting the ternary complex by detecting the exogenous detectable label of the labeled polymerase. For example, the exogenous detectable label of the labeled polymerase need not be a conformationally sensitive label. Here, each of the different nucleotides of the combinations in step (b) can be, for example, a different native nucleotide. In some embodiments, when step (b) involves serially contacting the primed template nucleic acid molecule with the plurality of polymerase-nucleotide combinations in the presence of the catalytic metal ion and in the absence of any non-catalytic ion that inhibits incorporation, each different nucleotide of the combination can be a different labeled nucleotide having an exogenous detectable label that produces a signal, where the signal produced by the different labeled nucleotides is substantially the same before and after formation of the ternary complex. In some other embodiments, when step (b) involves serially contacting the primed template nucleic acid molecule with the plurality of polymerase-nucleotide combinations in the presence of the catalytic metal ion and in the absence of any non-catalytic ion that inhibits incorporation, the primed template nucleic acid molecule can be contained within a flow cell, and step (b) can involve contacting the primed template nucleic acid molecule by flowing through the flow cell, one at a time, a plurality of reagent solutions, each including one of the plurality of polymerase-nucleotide combinations. In one preferred embodiment, the primed template nucleic acid molecule is disposed on a bead contained within the flow cell. According to a different preferred embodiment, the

method further includes the step of flowing through the flow cell, between flows of each of the plurality of reagent solutions, a wash buffer that removes any of the polymerase and the different nucleotides that did not combine with the primed template nucleic acid molecule to form the ternary complex. According to still a different preferred embodiment, the method further includes the step of (d) flowing through the flow cell a stripping buffer that dissociates the ternary complex to remove the polymerase and the one of the different nucleotides from the primed template nucleic acid molecule. Preferably, the disclosed method further involves removing the reversible terminator moiety attached to the 3' terminal nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule including an extendable primer. Still more preferably, the method further includes the step of (e) incorporating a nucleotide into the extendable primer. When this is the case, step (e) can involve incorporating with a polymerase different from the polymerase of step (a). Alternatively, the nucleotide incorporated into the extendable primer can include a reversible terminator moiety, and the incorporation produces a blocked primed template nucleic acid molecule. According to another generally preferred embodiment, the polymerase is a labeled polymerase including an exogenous detectable label. According to another generally preferred embodiment, each of the different nucleotides of the combinations is either a different native nucleotide, or a different unlabeled nucleotide analog that is free of any exogenous fluorescent moiety. According to another generally preferred embodiment, each different nucleotide of the combination is a different labeled nucleotide including an exogenous detectable label that produces a signal, and wherein the signal produced by each of the different labeled nucleotides is substantially the same before and after formation of the ternary complex. According to another generally preferred embodiment, the plurality of polymerase-nucleotide combinations consists of four polymerase-nucleotide combinations. According to another generally preferred embodiment, the primed template nucleic acid molecule is contained within a flow cell, and wherein step (b) includes serially contacting the primed template nucleic acid molecule by flowing through the flow cell, one at a time, a plurality of reagent solutions, each including one of the plurality of polymerase-nucleotide combinations. According to another generally preferred embodiment, the method can further include the step of removing the reversible terminator moiety from the 3' nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule including an extendable primer. According to another generally preferred embodiment, the method further includes the step of (d) flowing through the flow cell a stripping buffer that dissociates the ternary complex to

remove the polymerase and the one of the different nucleotides from the primed template nucleic acid molecule. More preferably, the method further includes the step of: (e) removing the reversible terminator moiety attached to the 3' terminal nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule that includes an extendable primer. Still more preferably, the method further includes the step of: (f) incorporating a nucleotide into the extendable primer. Yet more preferably, the nucleotide incorporated into the extendable primer can include a reversible terminator moiety. Yet still more preferably, the method can further involve repeating steps (a)-(f) at least once to obtain sequence information for the template nucleic acid molecule.

## Detailed Description

**[0044]** Disclosed herein is a technique that can be used for determining the nucleotide sequence of a primed template nucleic acid molecule, where identification of the next correct nucleotide in the sequence is completely independent of nucleotide incorporation. More particularly, the disclosed sequencing-by-binding approach relies on formation and detection of a ternary complex to identify the next correct nucleotide. This is distinct from sequencing-by-synthesis procedures that rely on identifying the incorporated nucleotide to determine sequence information.

**[0045]** The sequencing-by-binding procedure includes an "examination" step that identifies the next template base, and an optional "incorporation" step that adds one or more complementary nucleotides to the 3'-end of the primer. The incorporation step may be concurrent with or separate from the examination step. The examination step involves monitoring the interaction between a polymerase and a nucleic acid to be sequenced (template nucleic acid) in the presence of nucleotides. Optionally, the interaction may be in the presence of stabilizers, whereby the polymerase-nucleic acid interaction is stabilized in the presence of the next correct nucleotide. Alternatively, a primer having a blocked 3' terminal nucleotide may be used to stabilize the ternary complex and prevent the incorporation reaction from proceeding. Binary complexes between a primed template nucleic acid and a polymerase advantageously can be destabilized by a number of different approaches, such as providing conditions of high monovalent cation concentration, a concentration of glutamate ion, and combinations thereof.

**[0046]** Advantageously, the technique can be practiced using various types of nucleotides, including unlabeled (e.g., native) nucleotides, nucleotides with detectable labels (e.g., fluorescent or other optically detectable labels), or labeled or unlabeled nucleotide analogs

(e.g., modified nucleotides containing reversible terminator moieties). Further, the technique provides controlled reaction conditions, unambiguous determination of sequence, low overall cost of reagents, and low instrument cost.

**[0047]** The disclosed technique can be applied to binding reactions used for determining the identity of the next base of a primed template nucleic acid by any means and for any reason. The technique can be used to monitor specific binding of a DNA polymerase and the next correct nucleotide (e.g., a dNTP) complementary to a primed template nucleic acid, and to distinguish specific binding from nonspecific binding. The technique may be applied to single nucleotide determination (e.g., SNP determination), or alternatively to more extensive nucleic acid sequencing procedures employing reiterative cycles that identify one nucleotide at a time.

**[0048]** The compositions, systems, and methods provided herein overcome or reduce one or more problems associated with current sequencing-by-synthesis methods. The provided methods can even be carried out using native nucleotides that lack any exogenous detectable label. Optionally, the method is carried out in the absence of any detectable labels (e.g., on the nucleotides, polymerase or templates being sequenced). Of course, labeled nucleotides and/or labeled polymerases also can be used in disclosed procedure.

Definitions

**[0049]** 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. For clarity, the following specific terms have the specified meanings. Other terms are defined in other sections herein.

**[0050]** The singular forms "a" "an" and "the" include plural referents unless the context clearly dictates otherwise. Approximating language, as used in the description and claims, may be applied to modify any quantitative representation that could permissibly vary without resulting in a change in the basic function to which it is related. Accordingly, a value modified by a term such as "about" is not to be limited to the precise value specified. Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least each numerical parameter

should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

**[0051]** As used herein, the term "each," when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection. Exceptions can occur if explicit disclosure or context clearly dictates otherwise.

**[0052]** As used herein, "sequencing-by-binding" refers to a sequencing technique wherein specific binding of a polymerase and cognate nucleotide to a primed template nucleic acid is used for identifying the next correct nucleotide to be incorporated into the primer strand of the primed template nucleic acid. The specific binding interaction need not result in chemical incorporation of the nucleotide into the primer. In some embodiments, the specific binding interaction precedes chemical incorporation of the nucleotide into the primer strand or precedes chemical incorporation of an analogous, next correct nucleotide into the primer. Thus, identification of the next correct nucleotide can take place without incorporation of the next correct nucleotide.

**[0053]** As used herein, "stabilize" and its grammatical variants means to hold steady or limit fluctuations. "Stabilizing" a ternary complex refers to the process of promoting existence of the ternary complex and of preventing incorporation of a nucleotide. The term can be applied to any of a variety of complexes including, but not limited to a binary complex or ternary complex. For example, the complex that is stabilized can be a ternary complex between a polymerase, primed template nucleic acid molecule and cognate nucleotide. Generally, stabilization of the ternary complex prevents incorporation of the nucleotide component of the ternary complex into the primed nucleic acid component of the ternary complex. Accordingly, stabilizing a ternary complex can refer to promoting or prolonging non-covalent interactions that bind components of the ternary complex, or inhibiting disruption of non-covalent interactions that bind components of the ternary complex.

**[0054]** As used herein, "destabilize" and its grammatical variants means to cause something to be unable to continue existing or working in its usual way. "Destabilizing" a binary complex refers to the process of promoting dissolution or breakdown of the binary complex. "Destabilizing" also includes the process of inhibiting or preventing formation of the binary complex.

**[0055]** As used herein, a "salt providing monovalent cations" is an ionic compound that dissociates in aqueous solution to produce cations having a single positive charge. For example, the cations can be metal cations where the oxidation state +1.

**[0056]** As used herein, "a glutamate salt" is an ionic compound that dissociates in aqueous solution to produce glutamate anions.

**[0057]** As used herein, to provide reaction conditions that "enhance" ternary complex formation over binary complex formation means to provide conditions that give a ratio of ternary complex to binary complex signals that is greater than one. An enhancement of two-fold means that signal associated with ternary complex formation is twice the signal associated with binary complex formation.

**[0058]** As used herein, "nucleic acid" or "oligonucleotide" or "polynucleotide" means at least two nucleotides, or analogs thereof, covalently linked together. Thus, "nucleic acid" embraces DNA, RNA, or any combination thereof, that can be acted upon by a polymerizing enzyme during nucleic acid synthesis. The term includes single-, double-, or multiple-stranded DNA, RNA and analogs (derivatives) thereof. Double-stranded nucleic acids advantageously can minimize secondary structures that may hinder nucleic acid synthesis. A double stranded nucleic acid may possess a nick or a single-stranded gap. A nucleic acid may represent a single, plural, or clonally amplified population of nucleic acid molecules.

**[0059]** As used herein, a "template nucleic acid" is a nucleic acid to be detected, sequenced, evaluated or otherwise analyzed using any disclosed method.

**[0060]** As used herein, "primed template nucleic acid" is a template nucleic acid primed with (i.e., hybridized to) a primer, wherein the primer is an oligonucleotide having a 3'-end with a sequence complementary to at least a portion of the template nucleic acid. The primer can optionally have a free 5'-end (e.g., the primer being noncovalently associated with the template) or the primer can be continuous with the template (e.g., via a hairpin structure). The primed template nucleic acid includes the complementary primer bound to the template nucleic acid.

**[0061]** As used herein, the "next template nucleotide" (or the "next template base") refers to the next nucleotide (or base) in a template nucleic acid that is located immediately downstream of the 3'-end of a hybridized primer. In other words, the next template nucleotide (or base) is located in the template strand immediately 5' of the base in the template strand that is hybridized to the 3' end of the primer.

**[0062]** As used herein, the "next correct nucleotide" (sometimes referred to as the "cognate" nucleotide) is the nucleotide having a base complementary to the next template base located immediately downstream of the 3'-end of a hybridized primer. A nucleotide having a base that is not complementary to the next template base is referred to as an "incorrect" (or "non-cognate") nucleotide.

**[0063]** As used herein, a "blocked primed template nucleic acid" is a primed template nucleic acid modified to preclude or prevent phosphodiester bond formation at the 3'-end of the primer. Blocking may be accomplished, for example, by chemical modification with a blocking group at either the 3' or 2' position of the five-carbon sugar at the 3' terminus of the primer. Alternatively, or in addition, chemical modifications that preclude or prevent phosphodiester bond formation may also be made to the nitrogenous base of a nucleotide. Reversible terminator nucleotide analogs including each of these types of blocking groups will be familiar to those having an ordinary level of skill in the art. Incorporation of these analogs at the 3' terminus of a primer results in a blocked primed template nucleic acid.

**[0064]** As used herein, a "nucleotide" is a molecule that includes a nitrogenous base, a five-carbon sugar (ribose or deoxyribose), and at least one phosphate group. The term embraces ribonucleotides, deoxyribonucleotides, nucleotides modified to include exogenous labels or reversible terminators, and nucleotide analogs.

**[0065]** As used herein, a "test nucleotide" is a nucleotide being investigated for its ability to participate in formation of a ternary complex that further includes a primed template nucleic acid and a polymerase.

**[0066]** As used herein, a "native" nucleotide refers to a naturally occurring nucleotide that does not include an exogenous label (e.g., a fluorescent dye, or other label) or chemical modification such as may characterize a nucleotide analog. Examples of native nucleotides useful for carrying out the sequencing-by-binding procedures described herein include: dATP (2'-deoxyadenosine-5'-triphosphate); dGTP (2'-deoxyguanosine-5'-triphosphate); dCTP (2'-deoxycytidine-5'-triphosphate); dTTP (2'-deoxythymidine-5'-triphosphate); and dUTP (2'-deoxyuridine-5'-triphosphate).

**[0067]** As used herein, a "nucleotide analog" has modifications, such as chemical moieties, which replace and/or modify any of the components (e.g., nitrogenous base, five-carbon sugar, or phosphate group(s)) of a native nucleotide. Nucleotide analogs may be either incorporable or non-incorporable by a polymerase in a nucleic acid polymerization reaction. Optionally, the 3'-OH group of a nucleotide analog is modified with a moiety. The moiety may be a 3' reversible or irreversible terminator. The base of a nucleotide may be any of adenine, cytosine, guanine, thymine, or uracil, or analogs thereof. Optionally, a nucleotide has an inosine, xanthine, hypoxanthine, isocytosine, isoguanine, nitropyrrole (including 3-nitropyrrole) or nitroindole (including 5-nitroindole) base. Nucleotides may include, but are not limited to, ATP, UTP, CTP, GTP, ADP, UDP, CDP, GDP, AMP, UMP, CMP, GMP, dATP, dTTP, dUTP, dCTP, dGTP, dADP, dTDP, dCDP, dGDP, dAMP, dTMP, dCMP, and

dGMP. Nucleotides may also contain terminating inhibitors of DNA polymerase, dideoxynucleotides or 2',3' dideoxynucleotides, which are abbreviated as ddNTPs (ddGTP, ddATP, ddTTP, ddUTP and ddCTP).

**[0068]** As used herein, a "blocking moiety," when used with reference to a nucleotide analog, is a part of the nucleotide that inhibits or prevents the 3' oxygen of the nucleotide from forming a covalent linkage to a second nucleotide (e.g., via the 3'- oxygen of the nucleotide analog when it is present at the 3' end of a primer) during the incorporation step of a nucleic acid polymerization reaction. The blocking moiety of a "reversible terminator" nucleotide can be removed from the nucleotide analog to allow for nucleotide incorporation. Such a blocking moiety is referred to herein as a "reversible terminator moiety."

**[0069]** As used herein, a "polymerase" can refer to a nucleic acid synthesizing enzyme, including but not limited to, DNA polymerase, RNA polymerase, reverse transcriptase, primase and transferase. Typically, the polymerase includes one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization may occur. The polymerase may catalyze the polymerization of nucleotides to the 3'-end of a primer bound to its complementary nucleic acid strand. For example, a polymerase catalyzes the addition of a next correct nucleotide to the 3'-OH group of the primer via a phosphodiester bond, thereby chemically incorporating the nucleotide into the primer. Optionally a polymerase lacks catalytic nucleotide polymerization function, for example, due to a modification such as a mutation or chemical modification. Optionally, the polymerase used in the provided methods is a processive polymerase. Optionally, the polymerase used in the provided methods is a distributive polymerase.

**[0070]** As used herein, "providing" a template, a primer, a primed template nucleic acid, or a blocked primed template nucleic acid refers to the preparation or delivery of one or many nucleic acid polymers, for example to a reaction mixture or reaction chamber.

**[0071]** As used herein, "monitoring" (or sometimes "measuring") refers to a process of detecting a measurable interaction or binding between two molecular species. For example, monitoring may involve detecting measurable interactions between a polymerase and primed template nucleic acid, typically at various points throughout a procedure. Monitoring can be intermittent (e.g., periodic) or continuous (e.g., without interruption), and can involve acquisition of quantitative results. Monitoring can be carried out by detecting multiple signals over a period of time during a binding event or, alternatively, by detecting signal(s) at a single time point during or after a binding event.

**[0072]** As used herein, "contacting" refers to the mixing together of reagents (e.g., mixing an immobilized template nucleic acid and either a buffered solution that includes a polymerase, or the combination of a polymerase and a test nucleotide) so that a physical binding reaction or a chemical reaction may take place.

**[0073]** As used herein, "incorporating" or "chemically incorporating," when used in reference to a primer and cognate nucleotide, refers to the process of joining the cognate nucleotide to the primer by formation of a phosphodiester bond.

**[0074]** As used herein, a "binary complex" is a complex between a polymerase and a primed template nucleic acid, where the complex does not include the next correct nucleotide.

**[0075]** As used herein, a "ternary complex" is a complex between a polymerase, a primed template nucleic acid (e.g., having a primer with a free 3'-OH or a blocked 3' position), and the next correct nucleotide positioned immediately downstream of the primer and complementary to the template strand of the primed template nucleic acid. The primed template nucleic acid can include, for example, a primer with a free 3'-OH or a blocked primer (e.g., a primer with a chemical modification on the base or the sugar moiety of the 3' terminal nucleotide, where the modification precludes enzymatic phosphodiester bond formation).

**[0076]** As used herein, a "catalytic metal ion" refers to a metal ion required for phosphodiester bond formation between the 3'-OH of a nucleic acid (e.g., a primer) and the phosphate of an incoming nucleotide. A "divalent catalytic metal cation" is a catalytic metal ion having a valence of two. Catalytic metal ions can be present at concentrations necessary to stabilize formation of a complex between a polymerase, a nucleotide, and a primed template nucleic acid, referred to as non-catalytic concentrations of a metal ion. Catalytic concentrations of a metal ion refer to the amount of a metal ion needed by polymerases to catalyze the reaction between the 3'-OH group of a nucleic acid (e.g., a primer) and the phosphate group of an incoming nucleotide.

**[0077]** As used herein, a "non-catalytic metal ion" refers to a metal ion that, when in the presence of a polymerase enzyme, does not facilitate phosphodiester bond formation needed for chemical incorporation of a nucleotide into a primer. Typically, the non-catalytic metal ion is a cation. A non-catalytic metal ion may inhibit phosphodiester bond formation by a polymerase, and so may stabilize a ternary complex by preventing nucleotide incorporation. Non-catalytic metal ions may interact with polymerases, for example, via competitive binding compared to catalytic metal ions. A "divalent non-catalytic metal ion" is a non-catalytic metal ion having a valence of two. Examples of divalent non-catalytic metal ions

include, but are not limited to, Ca2+, Zn2+, Co2+, Ni2+, and Sr2+. The trivalent Eu3+ ion is a non-catalytic metal ion having a valence of three.

**[0078]** As used herein an "exogenous label" refers to a detectable chemical moiety that has been added to a sequencing reagent, such as a nucleotide or a polymerase (e.g., a DNA polymerase). While a native dNTP may have a characteristic limited fluorescence profile, the native dNTP does not include any added colorimetric or fluorescent moiety. Conversely, a dATP (2'-deoxyadenosine-5'-triphosphate) molecule modified to include a chemical linker and fluorescent moiety attached to the gamma phosphate would be said to include an exogenous label because the attached chemical components are not ordinarily a part of the nucleotide. Of course, chemical modifications to add detectable labels to nucleotide bases also would be considered exogenous labels. Likewise, a DNA polymerase modified to include a fluorescent dye (e.g., by attachment to a cys residue that is part of the primary sequence of the enzyme) also would be said to include an exogenous label because the label is not ordinarily a part of the polymerase.

**[0079]** As used herein, a "polymerase-nucleotide combination" refers to a polymerase and a nucleotide or nucleotide analog that are used together (*e.g.*, being mixed together and delivered as a mixture or combination), where both components are required for the combination.

**[0080]** As used herein, "discriminating conditions," when used in reference to polymerase complexes, are reaction conditions that distinguish between formation of a binary complex (a complex between the polymerase and a primed template nucleic acid molecule absent a cognate nucleotide) and a formation of a ternary complex (a complex between the polymerase, primed template nucleic acid molecule and a cognate nucleotide). Discriminating conditions may be provided by a number of routes, including: use of salts (*e.g.*, salts providing monovalent cations, or glutamate anions), use of polymerase enzymes engineered to exhibit low background binding in the presence of a non-cognate nucleotide, temperature adjustment, and/or pH adjustment *etc.*

**[0081]** As used herein, taking place "serially" or "in serial fashion" means taking place sequentially, one after the other. In some embodiments, two steps can occur in a series allowing for intervening steps or actions (*i.e.*, not necessarily without interruption). Thus, contacting a nucleic acid molecule with two different polymerase-nucleotide combinations "serially" or "in serial fashion" means contacting with the first combination, and then contacting with the second combination. Optionally, polymerase-nucleotide combinations that serially contact a primed template nucleic acid do not mingle with each other.

**[0082]** As used herein, a "flow cell" is a reaction chamber that includes one or more channels that direct fluid in a predetermined manner to conduct a desired reaction. The flow cell can be coupled to a detector such that a reaction occurring in the reaction chamber can be observed. For example, a flow cell can contain primed template nucleic acid molecules (or blocked primed template nucleic acid molecules), for example, tethered to a solid support, to which nucleotides and ancillary reagents are iteratively applied and washed away. The flow cell can include a transparent material that permits the sample to be imaged after a desired reaction occurs. For example, a flow cell can include a glass slide containing small fluidic channels, through which polymerases, dNTPs and buffers can be pumped. The glass inside the channels is decorated with one or more primed template nucleic acid molecules to be sequenced. An external imaging system can be positioned to detect the molecules on the surface of the glass. Reagent exchange in a flow cell is accomplished by pumping, drawing, or otherwise "flowing" different liquid reagents through the flow cell. Exemplary flow cells, methods for their manufacture and methods for their use are described in US Pat. App. Publ. Nos. 2010/0111768 A1 or 2012-0270305 A1; or WO 05/065814, each of which is incorporated by reference herein.

**[0083]** As used herein, "unlabeled" refers to a molecular species free of added or exogenous label(s) or tag(s). A native nucleotide is an example of an unlabeled molecular species.

Sequencing-by-Binding

**[0084]** Described herein, are polymerase-based, nucleic acid sequencing-by-binding reactions, wherein the polymerase undergoes conformational transitions between open and closed conformations during discrete steps of the reaction. In one step, the polymerase binds to a primed template nucleic acid to form a binary complex, also referred to herein as the pre-insertion conformation. In a subsequent step, an incoming nucleotide is bound and the polymerase fingers close, forming a pre-chemistry conformation comprising the polymerase, primed template nucleic acid and nucleotide; wherein the bound nucleotide has not been incorporated. This step, also referred to herein as an examination step, may be followed by a chemical incorporation step wherein a phosphodiester bond is formed with concomitant pyrophosphate cleavage from the nucleotide (nucleotide incorporation). The polymerase, primed template nucleic acid and newly incorporated nucleotide produce a post-chemistry, pre-translation conformation. As both the pre-chemistry conformation and the pre-translocation conformation comprise a polymerase, primed template nucleic acid and nucleotide, wherein the polymerase is in a closed state, either conformation may be referred

to herein as a ternary complex. Notably however, phosphodiester bond formation can be precluded by the approach described herein, so that the referenced ternary complexes are necessarily in the pre-chemistry conformation. In the closed pre-insertion state, divalent catalytic metal ions, such as Mg2+ mediate a rapid chemical step involving nucleophilic displacement of a pyrophosphate (PPi) by the 3'-hydroxyl of the primer terminus. The polymerase returns to an open state upon the release of PPi, the post-translocation step, and translocation initiates the next round of reaction. While a ternary complex can form in the absence of a divalent catalytic metal ions (e.g., Mg2+), it is proficient in chemical addition of nucleotide in the presence of the divalent metal ions. Low or deficient levels of catalytic metal ions, such as Mg2+ tend to lead to non-covalent (physical) sequestration of the next correct nucleotide in a tight ternary complex. This ternary complex may be referred to as a stabilized or trapped ternary complex. In any reaction step described above, the polymerase configuration and/or interaction with a nucleic acid may be monitored during an examination step to identify the next correct base in the nucleic acid sequence. Before or after incorporation, reaction conditions can be changed to disengage the polymerase from the primed template nucleic acid, and changed again to remove from the local environment any reagents that inhibit polymerase binding.

**[0085]** Generally speaking, the SBB procedure includes an "examination" step that identifies the next template base, and optionally an "incorporation" step that adds one or more complementary nucleotides to the 3'-end of the primer component of the primed template nucleic acid. Identity of the next correct nucleotide to be added is determined either without, or before chemical linkage of that nucleotide to the 3'-end of the primer through a covalent bond. The examination step can involve providing a primed template nucleic acid to be used in the procedure, and contacting the primed template nucleic acid with a polymerase enzyme (e.g., a DNA polymerase) and one or more test nucleotides being investigated as the possible next correct nucleotide. Further, there is a step that involves monitoring or measuring the interaction between the polymerase and the primed template nucleic acid in the presence of the test nucleotides. Optionally, the interaction can take place when the primer of the primed template nucleic acid molecule includes a blocking group that precludes enzymatic incorporation of an incoming nucleotide into the primer. Optionally, the interaction can take place in the presence of stabilizers, whereby the polymerase-nucleic acid interaction is stabilized in the presence of the next correct nucleotide (i.e., stabilizers that stabilize the ternary complex). Again, the examination step identifies or determines the identity of the next correct nucleotide without requiring incorporation of that nucleotide. Stated differently,

identity of the next correct nucleotide can be established without chemical incorporation of the nucleotide into the primer when one or more cycles of examination is carried out using labeled or unlabeled nucleotides. Likewise, the polymerase employed in the procedure can be labeled or unlabeled.

**[0086]** Whereas methods involving a single template nucleic acid molecule may be described for convenience, these methods are exemplary. The sequencing methods provided herein readily encompass a plurality of template nucleic acids, wherein the plurality of nucleic acids may be clonally amplified copies of a single nucleic acid, or disparate nucleic acids, including combinations, such as populations of disparate nucleic acids that are clonally amplified.

The Examination Step

**[0087]** An examination step typically includes the following substeps: (1) providing a primed template nucleic acid (i.e., a template nucleic acid molecule hybridized with a primer that optionally may be blocked from extension at its 3'-end); (2) contacting the primed template nucleic acid with a reaction mixture that includes a polymerase and at least one nucleotide; (3) monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of the nucleotide(s) and without chemical incorporation of any nucleotide into the primed template nucleic acid; and (4) determining from the monitored interaction the identity of the next base in the template nucleic acid (i.e., the next correct nucleotide). Optionally, the primed template nucleic acid molecule can be contacted initially with the polymerase in the absence of nucleotide(s) before contacting any nucleotide. The primer of the primed template nucleic acid can be an extendible primer. The primed template nucleic acid, the polymerase and the test nucleotide are capable of forming a ternary complex when the base of the test nucleotide is complementary to the next base of the primed template nucleic acid molecule. The primed template nucleic acid and the polymerase are capable of forming a binary complex when the base of the test nucleotide is not complementary to the next base of the primed template nucleic acid molecule. Optionally, the contacting occurs under conditions that favor formation of the ternary complex over formation of the binary complex. Optionally, the conditions that favor or stabilize the ternary complex are provided by either: (1) the presence of a reversible terminator moiety on the 3' nucleotide of the primer of the primed template nucleic acid molecule; or (2) the presence of a non-catalytic ion (e.g., a divalent non-catalytic metal ion). Optionally, the conditions that disfavor or destabilize binary complexes are provided by the presence of one or more monovalent cations and/or

glutamate anions in the reaction mixture during the examination step. The determining or identifying step can include identifying the base of the nucleotide that is complementary to the next base of the primed template nucleic acid. Optionally, this includes contacting ternary complexes with one or more wash solutions having different nucleotide compositions that permit ternary complexes to be selectively maintained or dissociated.

**[0088]** The examination step may be controlled so that nucleotide incorporation is either attenuated or accomplished. If nucleotide incorporation is attenuated, a separate incorporation step may be performed. The separate incorporation step may be accomplished without the need for monitoring, as the base has already been identified during the examination step. If nucleotide incorporation proceeds during examination, subsequent nucleotide incorporation may be attenuated by a stabilizer that traps the polymerase on the nucleic acid after incorporation. A reversibly terminated nucleotide may be used in the incorporation step to prevent the addition of more than one nucleotide during a single cycle.

**[0089]** The sequencing-by-binding method allows for controlled determination of a template nucleic acid base without the need for labeled nucleotides, as the interaction between the polymerase and template nucleic acid can be monitored without a label on the nucleotide. The controlled nucleotide incorporation can also provide accurate sequence information of repetitive and homopolymeric regions without necessitating use of a labeled nucleotide. Moreover, template nucleic acid molecules may be sequenced under examination conditions which do not require attachment of template nucleic acid or polymerase to a solid-phase support. However, in certain preferred embodiments, primed template nucleic acids to be sequenced are attached to a solid support, such as an interior surface of a flow cell. The compositions, methods and systems described herein provide numerous advantages over previous systems, such as controlled reaction conditions, unambiguous determination of sequence, long read lengths, low overall cost of reagents, and low instrument cost.

**[0090]** Further provided herein is a method for sequencing a template nucleic acid molecule, including an examination step that includes providing a template nucleic acid molecule primed with a primer (i.e., a primed template nucleic acid molecule); contacting the primed template nucleic acid molecule with a first reaction mixture that includes a polymerase and a first nucleotide molecule, wherein the primed template nucleic acid molecule, the polymerase and the first nucleotide molecule are capable of forming a ternary complex when the first nucleotide molecule is complementary to a next base of the primed template nucleic acid molecule, and wherein the primed template nucleic acid molecule and the polymerase are capable of forming a binary complex when the first nucleotide molecule is not

complementary to a next base of the primed template nucleic acid molecule. The method further includes monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of the first nucleotide molecule, and without chemical incorporation of the first nucleotide molecule into the primer of the primed template nucleic acid molecule; and determining the identity of the nucleotide that is complementary to the next base of the primed template nucleic acid molecule by the monitored interaction. Optionally, the contacting occurs under conditions that stabilize formation of the ternary complex and destabilize formation of the binary complex. Optionally, the conditions that stabilize the ternary complex are provided by either: (1) the presence of a reversible terminator moiety on the 3' nucleotide of the primer of the primed template nucleic acid molecule; or (2) the presence of a non-catalytic ion (e.g., a divalent non-catalytic metal ion). Optionally, the conditions that destabilize binary complexes are provided by the presence of one or more monovalent cations and/or glutamate anions in the reaction mixture during the examination step. These steps can be repeated one or more times. For example, the contacting and monitoring steps can be repeated one or more times. Optionally, the contacting and monitoring steps are repeated using the first reaction mixture. Optionally, the contacting and monitoring steps are repeated using a second reaction mixture including the polymerase and a second nucleotide molecule. Optionally, the contacting and monitoring steps are repeated using a third reaction mixture including the polymerase and a third nucleotide molecule. Optionally, the contacting and monitoring steps are repeated using a fourth reaction mixture including the polymerase and a fourth nucleotide molecule.

**[0091]** Also provided is a method for sequencing a template nucleic acid molecule. The method includes an examination step that involves providing a template nucleic acid molecule primed with a primer (i.e., a primed template nucleic acid molecule); contacting the primed template nucleic acid molecule with a reaction mixture including an polymerase, a first nucleotide molecule and a second nucleotide molecule, the first and second nucleotide molecules being different from each other and present simultaneously in the reaction mixture, optionally at different concentrations, wherein the primed template nucleic acid molecule, the polymerase and the first and/or second nucleotide molecule are capable of forming a ternary complex when the first and/or second nucleotide molecule is complementary to a next base of the primed template nucleic acid molecule, wherein the primed template nucleic acid molecule and the polymerase are capable of forming a binary complex when the first and/or second nucleotide molecule is not complementary to a next base of the primed template nucleic acid molecule. Optionally, the contacting occurs under conditions that stabilize

formation of the ternary complex, and destabilize formation of the binary complex. Optionally, the conditions stabilize formation of the ternary complex and destabilize formation of the binary complex. Optionally, the conditions that stabilize the ternary complex are provided by either: (1) the presence of a reversible terminator moiety on the 3' nucleotide of the primer of the primed template nucleic acid molecule; or (2) the presence of a non-catalytic ion (e.g., a divalent non-catalytic metal ion). Optionally, the conditions that destabilize binary complexes are provided by the presence of one or more monovalent cations and/or glutamate anions in the reaction mixture during the examination step. The method also includes monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of the first and second nucleotide molecules, and without chemical incorporation of either of the first or second nucleotide molecules into the primer of the primed template nucleic acid molecule; and determining whether any of the nucleotides includes a base complementary to the next base of the primed template nucleic acid molecule by the monitored interaction. Optionally, the reaction mixture further includes a third nucleotide molecule, wherein the third nucleotide molecule is different from the first and second nucleotide molecules and present in the reaction mixture at a different concentration than the first and second nucleotide molecules. Optionally, the reaction mixture further includes a fourth nucleotide molecule, wherein the fourth nucleotide molecule is different from the first, second and third nucleotide molecules and present in the reaction mixture at a different concentration than the first, second and third nucleotide molecules. Optionally, the first reaction mixture includes one or more first nucleotide molecules capable of incorporation and one or more first nucleotide molecules incapable of incorporation into the primer of the primed template nucleic acid molecule. Optionally, the second reaction mixture comprises one or more second nucleotide molecules capable of incorporation and one or more second nucleotide molecules incapable of incorporation into the primer of the primed template nucleic acid molecule. Optionally, the third reaction mixture comprises one or more third nucleotide molecules capable of incorporation and one or more third nucleotide molecules incapable of incorporation into the primer of the primed template nucleic acid molecule. Optionally, the fourth reaction mixture comprises one or more fourth nucleotide molecules capable of incorporation and one or more fourth nucleotide molecules incapable of incorporation into the primer of the primed template nucleic acid molecule.

**[0092]** Optionally, the provided methods further include a wash step. The wash step can occur before or after any other step in the method. Optionally, the wash step is performed prior to the monitoring step and/or prior to the determining or identifying step. Optionally,

the wash step occurs under conditions that stabilize the ternary complex. Optionally, the conditions result from the presence of a reversible terminator moiety on the 3' nucleotide of the primer of the primed template nucleic acid molecule. Optionally, the conditions include a stabilizing agent. Optionally, the stabilizing agent is a non-catalytic metal ion (e.g., a divalent non-catalytic metal ion). Non-catalytic metal ions include, but are not limited to, calcium, strontium, scandium, titanium, vanadium, chromium, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, rhodium, europium, and terbium ions. Optionally, the non-catalytic metal ion is any of strontium, tin, or nickel ions. Optionally, the ternary complex has a half-life and the wash step is performed for a duration shorter than the half-life of the ternary complex formed when a nucleotide molecule provides a base that is complementary to the next base of the primed template nucleic acid molecule.

**[0093]** Optionally, there is a reloading step following the monitoring step. The reloading step includes contacting the primed template nucleic acid with a reloading mixture that includes the polymerase and the first or optional second, third and fourth nucleotide molecule under conditions that stabilize the ternary complex and destabilize binary complex formation.

**[0094]** The examination step may be controlled, in part, by providing reaction conditions to prevent chemical incorporation of a nucleotide, while allowing determination of the identity of the next correct base on the primed template nucleic acid molecule. Such reaction conditions may be referred to as examination reaction conditions. Optionally, a ternary complex is formed under examination conditions. Optionally, a stabilized ternary complex is formed under examination conditions or in a pre-chemistry conformation. Optionally, a stabilized ternary complex is in a pre-translocation conformation, wherein the enclosed nucleotide has been incorporated, but the ternary complex does not allow for the incorporation of a subsequent nucleotide.

**[0095]** Optionally, the examination conditions accentuate the difference in affinity for polymerase to primed template nucleic acids in the presence of different nucleotides, for example by destabilizing binary complexes. Optionally, the examination conditions cause differential affinity of the polymerase for the primed template nucleic acid in the presence of different nucleotides. By way of example, the examination conditions that cause differential affinity of the polymerase for the primed template nucleic acid in the presence of different nucleotides include, but are not limited to, high salt and glutamate ions. For example, the salt may dissolve in aqueous solution to yield a monovalent cation, such as a monovalent metal cation (e.g., sodium ion or potassium ion). Optionally, the salt that provides the monovalent cations (e.g., monovalent metal cations) further provides glutamate anions. The source of

glutamate ions can be potassium glutamate. In some instances, the concentrations of potassium glutamate that can be used to alter polymerase affinity of the primed template nucleic acid extend from 10 mM to 1.6 M of potassium glutamate, or any amount in between 10 mM and 1.6 M. Optionally, as indicated above, high salt refers to a concentration of salt from 50 to 1,500 mM salt.

**[0096]** Examination typically involves detecting polymerase interaction with a template nucleic acid. Detection may include optical, electrical, thermal, acoustic, chemical and mechanical means. Optionally, examination is performed after a wash step, wherein the wash step removes any non-bound reagents (e.g., unbound polymerases and/or nucleotides) from the region of observation. Optionally, examination is performed during a wash step, such that the dissociation kinetics of the polymerase-nucleic acid or polymerase-nucleic acid-nucleotide complexes may be used to determine the identity of the next base. Optionally, examination is performed during the course of addition of the examination reaction mixture (or first reaction mixture), such that the association kinetics of the polymerase to the nucleic acid may be used to determine the identity of the next base on the nucleic acid. Optionally, examination involves distinguishing ternary complexes from binary complexes of polymerase and nucleic acid. Optionally, examination is performed under equilibrium conditions where the affinities measured are equilibrium affinities. Multiple examination steps comprising different or similar examination reagents, may be performed sequentially to ascertain the identity of the next template base. Multiple examination steps may be utilized in cases where multiple template nucleic acids are being sequenced simultaneously in one sequencing reaction, wherein different nucleic acids react differently to the different examination reagents. Optionally, multiple examination steps may improve the accuracy of next base determination.

**[0097]** Generally, the examination step involves binding a polymerase to the polymerization initiation site of a primed template nucleic acid in a reaction mixture comprising one or more nucleotides, and monitoring the interaction. Optionally, a nucleotide is sequestered within the polymerase-primed template nucleic acid complex to form a ternary complex, under conditions in which incorporation of the enclosed nucleotide by the polymerase is attenuated or inhibited. Optionally, the ternary complex is stabilized by the presence of a blocking moiety (e.g., a reversible terminator moiety) on the 3' terminal nucleotide of the primer. Optionally a stabilizer is added to stabilize the ternary complex in the presence of the next correct nucleotide. This ternary complex is in a stabilized or polymerase-trapped pre-chemistry conformation. A ternary complex that allows for the incorporation of the enclosed

nucleotide but does not allow for the incorporation of a subsequent nucleotide is in a stabilized or trapped pre-translocation conformation. Optionally, the polymerase is trapped at the polymerization site in its ternary complex by one or a combination of means, not limited to, crosslinking of the polymerase domains, crosslinking of the polymerase to the nucleic acid, allosteric inhibition by small molecules, uncompetitive inhibitors, competitive inhibitors, non-competitive inhibitors, and denaturation; wherein the formation of the trapped ternary complex provides information about the identity of the next base on the nucleic acid template.

**[0098]** Generally speaking, in accordance with the methods provided herein, the polymerase interacts with the primed template nucleic acid molecule in the presence of the at least one nucleotide molecule to form a complex. Optionally, the nucleotide molecule is a nucleotide including a base complementary to a base of the template nucleic acid molecule that is downstream of a 3'-end of the primer in the primed template nucleic acid molecule. Optionally, the nucleotide molecule is a next correct nucleotide (i.e., includes a base complementary to a base of the template nucleic acid molecule that is downstream of a 3'-end of the primer in the primed template nucleic acid molecule), and the complex is a ternary complex that includes the primed template nucleic acid molecule, the polymerase, and the next correct nucleotide. Optionally, the formation of a ternary complex is favored over the formation of a binary complex between the primed template nucleic acid and the polymerase. Optionally, the contacting occurs under conditions that stabilize formation of the ternary complex and destabilize formation of the binary complex. Optionally, the conditions that stabilize the ternary complex are provided by either: (1) the presence of a reversible terminator moiety on the 3' nucleotide of the primer of the primed template nucleic acid molecule; or (2) the presence of a non-catalytic ion (e.g., a divalent non-catalytic metal ion). Optionally, the conditions that destabilize binary complexes are provided by the presence of one or more monovalent cations and/or glutamate anions in the reaction mixture during the examination step. In a preferred embodiment, the formation of the ternary complex may be favored over the formation of the binary complex when the first reaction mixture includes a high concentration of salt. Optionally, the first reaction mixture includes 50 mM to 1,500 mM of a salt to destabilize binary complex formation. Salt concentrations in the range of from 100 mM to 1,500 mM, and from 200 mM to 1,500 mM are highly preferred. In certain embodiments, the salt used for destabilizing binary complexes dissolves to yield a monovalent ion, such as a monovalent metal cation (e.g., sodium ion or potassium ion). In some instances, the salt is a glutamate salt that provides the monovalent metal cation and a

glutamate anion. Formation of the ternary complex alternatively may be favored over the formation of the binary complex when the first reaction mixture comprises a buffer having a high pH. Optionally, the pH is from 7.4 to 9.0. For certain polymerases extracted from extremophile environments, the formation of the ternary complex may be favored over the formation of binary complex when the first reaction mixture comprises a buffer having low pH. Optionally, the pH is from 4.0-7.0. The reaction temperature and/or organic and inorganic additives may also be used to modulate the affinity between the polymerase and primed template nucleic acid molecules.

**[0099]** In the sequencing methods provided herein, a reaction mixture used in the examination step can include 1, 2, 3, or 4 types of nucleotide molecules. Optionally, the nucleotides are selected from dATP, dTTP (or dUTP), dCTP, and dGTP. Optionally, the reaction mixture comprises one or more triphosphate nucleotides and one or more diphosphate nucleotides. Optionally, a ternary complex is formed between the primed template nucleic acid, the polymerase, and any one of the four nucleotide molecules so that four types of ternary complexes may be formed.

Contacting Steps

**[0100]** In the provided methods, contacting of the primed template nucleic acid molecule with a reaction mixture that includes a polymerase and one or more nucleotide molecules preferably occurs under conditions that stabilize formation of the ternary complex, and that destabilize formation of binary complexes. These conditions can be provided by alternative approaches that are a matter of choice by the end-user.

**[0101]** Optionally, the conditions comprise contacting the primed nucleic acid molecule with a buffer that regulates osmotic pressure. Optionally, the reaction mixture used in the examination step includes the buffer that regulates osmotic pressure. Optionally, the buffer is a high salt buffer that includes a monovalent ion, such as a monovalent metal ion (e.g., potassium ion or sodium ion) at a concentration of from 50 to 1,500 mM. Salt concentrations in the range of from 100 to 1,500 mM, and from 200 to 1,500 mM also are highly preferred. Optionally, the buffer further includes a source of glutamate ions (e.g., potassium glutamate). Optionally, the conditions that stabilize formation of the ternary complex involve contacting the primed nucleic acid molecule with a stabilizing agent. Optionally, the reaction mixture used during the examination step includes a stabilizing agent. Optionally, the stabilizing agent is a non-catalytic metal ion (e.g., a divalent non-catalytic metal ion). Non-catalytic metal ions useful in this context include, but are not limited to, calcium, strontium, scandium,

titanium, vanadium, chromium, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, rhodium, europium, and terbium. Optionally, Ni2+ is provided in an examination reaction to facilitate ternary complex formation. Optionally, Sr2+ is provided in an examination reaction to facilitate ternary complex formation. Optionally, the non-catalytic metal ion is strontium, tin, or nickel. Optionally, the first reaction mixture comprises from 0.01 mM to 30 mM strontium chloride or nickel chloride.

**[0102]** Optionally, the contacting step is facilitated by the use of a flow cell or chamber ("flow cell" hereafter). Flowing liquid reagents through the flow cell, which contains an interior solid support surface (e.g., a planar surface), conveniently permits reagent exchange. Immobilized to the interior surface of the flow cell is one or more primed template nucleic acids to be sequenced or interrogated using the procedures described herein. Typical flow cells will include microfluidic valving that permits delivery of liquid reagents (e.g., components of the "reaction mixtures" discussed herein) to an entry port. Liquid reagents can be removed from the flow cell by exiting through an exit port.

**[0103]** Optionally, the contacting step is facilitated by physical transfer or transport of a sensor from one reagent reservoir to another. Plastic multiwell plates can serve as the reagent reservoirs. For example, an optical sensor tip having primed template nucleic acid molecules immobilized thereon can be transported from one well of a multiwell plate to a different well of a multiwell plate with continuous or intermittent monitoring during the transport steps.

Monitoring Steps

**[0104]** Monitoring or measuring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of a nucleotide molecule may be accomplished in many different ways. For example, monitoring can include measuring association kinetics for the interaction between the primed template nucleic acid, the polymerase, and any one of the four nucleotide molecules. Monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of a nucleotide molecule can include measuring equilibrium binding constants between the polymerase and primed template nucleic acid molecule (i.e., equilibrium binding constants of polymerase to the template nucleic acid in the presence of any one or the four nucleotides). Thus, for example, the monitoring includes measuring the equilibrium binding constant of the polymerase to the primed template nucleic acid in the presence of any one of the four nucleotides. Monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of a nucleotide molecule includes measuring dissociation kinetics of the polymerase from the primed

template nucleic acid in the presence of any one of the four nucleotides. Optionally, monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of a nucleotide molecule includes measuring dissociation kinetics of the dissociation of the closed-complex (i.e., dissociation of the primed template nucleic acid, the polymerase, and any one of the four nucleotide molecules). Optionally, the measured association kinetics are different depending on the identity of the nucleotide molecule. Optionally, the polymerase has a different affinity for each of the four types of nucleotide molecules. Optionally, the polymerase has a different dissociation constant for each of the four types of nucleotide molecules in each type of ternary complex. Association, equilibrium and dissociation kinetics are known and can be readily determined by one in the art. See, for example, Markiewicz et al., Nucleic Acids Research 40(16):7975-84 (2012); Xia et al., J. Am. Chem. Soc. 135(1):193-202 (2013); Brown et al., J. Nucleic Acids, Article ID 871939, 11 pages (2010); Washington, et al., Mol. Cell. Biol. 24(2):936-43 (2004); Walsh and Beuning, J. Nucleic Acids, Article ID 530963, 17 pages (2012); and Roettger, et al., Biochemistry 47(37):9718-9727 (2008), which are incorporated by reference herein in their entireties.

**[0105]** The monitoring step can include monitoring the steady state interaction of the polymerase with the primed template nucleic acid molecule in the presence of the first nucleotide molecule, without chemical incorporation of the first nucleotide molecule into the primer of the primed template nucleic acid molecule. Optionally, the monitoring includes monitoring dissociation of the polymerase with the primed template nucleic acid molecule in the presence of the first nucleotide molecule, without chemical incorporation of the first nucleotide molecule into the primer of the primed template nucleic acid molecule. Optionally, the monitoring includes monitoring association of the polymerase with the primed template nucleic acid molecule in the presence of the first nucleotide molecule, without chemical incorporation of the first nucleotide molecule into the primer of the primed template nucleic acid molecule. Again, the test nucleotides in these procedures may be native nucleotides (i.e., unlabeled), labeled nucleotides (e.g., fluorescently labeled nucleotides), or nucleotide analogs (e.g., nucleotides modified to include reversible terminator moieties).

**[0106]** In the sequencing methods provided herein, either a chemical block on the 3' nucleotide of the primer of the primed template nucleic acid molecule (e.g., a reversible terminator moiety on the base or sugar of the nucleotide), or the absence of a catalytic metal ion in the reaction mixture, or the absence of a catalytic metal ion in the active site of the polymerase prevents the chemical incorporation of the nucleotide into the primer of the

primed template nucleic acid. Optionally, the chelation of a catalytic metal ion in the reaction mixtures of the contacting step prevents the chemical incorporation of the nucleotide into the primer of the primed template nucleic acid. Optionally, a non-catalytic metal ion acts as a stabilizer for the ternary complex in the presence of the next correct nucleotide. Optionally, the substitution of a catalytic metal ion in the reaction mixtures of the contacting step with a non-catalytic metal ion prevents the chemical incorporation of the nucleotide molecule to the primed template nucleic acid. Optionally, the catalytic metal ion is magnesium. The metal ion mechanisms of polymerases postulate that a low concentration of metal ions may be needed to stabilize the polymerase-nucleotide-DNA binding interaction. See, for instance, Section 27.2.2, Berg JM, Tymoczko JL, Stryer L, Biochemistry 5th Edition, WH Freeman Press, 2002.

**[0107]** Optionally, a low concentration of a catalytic ion in the reaction mixture used during the examination step prevents the chemical incorporation of the nucleotide molecule to the primed template nucleic acid. Optionally, a low concentration is from about 1 μM to about 100 μM. Optionally, a low concentration is from about 0.5 μM to about 5 μM. Optionally, the reaction mixture used during the examination step includes cobalt ions and the incorporating step involves contacting with an incorporation reaction mixture that includes a higher concentration of cobalt ions as compared to the concentration of cobalt ions in the first reaction mixture.

**[0108]** In an exemplary sequencing reaction, the examination step comprises formation and/or stabilization of a ternary complex comprising a polymerase, primed template nucleic acid, and nucleotide. Characteristics of the formation and/or release of the ternary complex are monitored to identify the enclosed nucleotide and therefore the next base in the template nucleic acid. Ternary complex characteristics can be dependent on the sequencing reaction components (e.g., polymerase, primer, template nucleic acid, nucleotide) and/or reaction mixture components and/or conditions. Optionally, the ternary complex is in a pre-chemistry conformation. Optionally, the ternary complex is in a pre-translocation conformation. Optionally, the ternary complex is in a post-translocation conformation.

**[0109]** The examination step involves monitoring the interaction of a polymerase with a template nucleic acid in the presence of a nucleotide. The formation of a ternary complex may be monitored. Optionally, the absence of formation of ternary complex is monitored. Optionally, the dissociation of a ternary complex is monitored. Optionally, the incorporation step involves monitoring incorporation of a nucleotide. Optionally, the incorporation step involves monitoring the absence of nucleotide incorporation.

**[0110]** Any process of the examination and/or incorporation step may be monitored. Optionally, a polymerase has a detectable tag. Optionally, no component of the sequencing reaction is detectably labeled. Optionally, the detectable tag or label on the polymerase is removable. Optionally, the nucleotides or polymerases have a detectable label, however, the label is not detected during sequencing.

**[0111]** Monitoring the variation in affinity of a polymerase to a template nucleic acid in the presence of correct and incorrect nucleotides, under conditions that may or may not allow the incorporation of the nucleotide, may be used to determine the sequence of the nucleic acid. The affinity of a polymerase to a template nucleic acid in the presence of different nucleotides, including modified or labeled nucleotides, can be monitored as the off-rate of the polymerase-nucleic acid interaction in the presence of the various nucleotides. The affinities and off-rates of many standard polymerases to various matched/correct, mismatched/incorrect and modified nucleotides are known in the art. Single molecule imaging of Klenow polymerase reveals that the off-rate for a template nucleic acid for different nucleotide types, where the nucleotide types are prevented from incorporating, are distinctly and measurably different.

**[0112]** Optionally, a nucleotide of a particular type is made available to a polymerase in the presence of a primed template nucleic acid. The reaction is monitored, wherein, if the nucleotide is a next correct nucleotide, the polymerase may be stabilized to form a ternary complex. If the nucleotide is an incorrect nucleotide, a ternary complex may still be formed; however, without the additional assistance of stabilizing agents or reaction conditions (e.g., absence of catalytic ions, polymerase inhibitors, salt), the ternary complex may dissociate. The rate of dissociation is dependent on the affinity of the particular combination of polymerase, template nucleic acid, and nucleotide, as well as reaction conditions. Optionally, the affinity is measured as an off-rate. Optionally, the affinity is different between different nucleotides for the ternary complex. For example, if the next base in the template nucleic acid downstream of the 3'-end of the primer is G, the polymerase-nucleic acid affinity, measured as an off-rate, is expected to be different based on whether dATP, dCTP, dGTP or dTTP are added. In this case, dCTP would have the slowest off-rate, with the other nucleotides providing different off-rates for the interaction. Optionally, the off-rate may be different depending on the reaction conditions, for example, the presence of stabilizing agents (e.g., absence of magnesium or inhibitory compounds) or reaction conditions (e.g., nucleotide modifications or modified polymerases).

**[0113]** Once the identity of the next correct nucleotide is determined, 1, 2, 3, 4 or more nucleotide types may be introduced simultaneously to the reaction mixture under conditions that specifically target the formation of a ternary complex. Excess nucleotides optionally may be removed from the reaction mixture and the reaction conditions modulated to incorporate the next correct nucleotide of the ternary complex. This sequencing reaction ensures that only one nucleotide is incorporated per sequencing cycle. Preferably, reversible terminator nucleotides are employed in the incorporation step, and the optional wash step is omitted.

Identifying Steps

**[0114]** The identity of the next correct base or nucleotide can be determined by monitoring the presence, formation, and/or dissociation of the ternary complex. The identity of the next base may be determined without chemically incorporating the next correct nucleotide to the 3'-end of the primer. Optionally, the identity of the next base is determined by monitoring the affinity of the polymerase to the primed nucleic acid template in the presence of added nucleotides. Optionally, the affinity of the polymerase to the primed template nucleic acid in the presence of the next correct nucleotide may be used to determine the next correct base on the template nucleic acid. Optionally, the affinity of the polymerase to the primed nucleic acid template in the presence of an incorrect nucleotide may be used to determine the next correct base on the template nucleic acid.

**[0115]** In certain embodiments, a ternary complex that includes a primed template nucleic acid (or a blocked primed template nucleic acid) is formed in the presence of a polymerase and a plurality of nucleotides. Cognate nucleotide participating in the ternary complex optionally is identified by observing destabilization of the complex that occurs when the cognate nucleotide is absent from the reaction mixture. This is conveniently carried out, for example, by exchanging one reaction mixture for another. Here, loss of the complex is an indicator of cognate nucleotide identity. Loss of binding signal (e.g., a fluorescent binding signal associated with a particular locus on a solid support) can occur when the primed template nucleic acid is exposed to a reaction mixture that does not include the cognate nucleotide. Optionally, maintenance of a ternary complex in the presence of a single nucleotide in a reaction mixture also can indicate identity of the cognate nucleotide.

Incorporation Steps

**[0116]** The sequencing methods described herein optionally include an incorporation step. The incorporation step involves chemically incorporating one or more nucleotides at the 3'-end of a primer bound to a template nucleic acid. Optionally, one or more nucleotides is incorporated at the 3'-end of the primer. In a preferred embodiment, only a single nucleotide is incorporated at the 3'-end of the primer. Optionally, multiple nucleotides of the same kind are incorporated at the 3'-end of the primer. Optionally, multiple nucleotides of different kinds are incorporated at the 3'-end of the primer. Incorporated nucleotides alternatively can be unlabeled nucleotides, reversible terminator nucleotides, or detectably labeled nucleotide analogs. The polymerase can dissociate from the polymerization initiation site after nucleotide incorporation or can be retained at the polymerization initiation site after incorporation.

**[0117]** The incorporation reaction may be facilitated by an incorporation reaction mixture. Optionally, the incorporation reaction mixture has a different composition of nucleotides than the examination reaction. For example, the examination reaction can include one type of nucleotide and the incorporation reaction can include another type of nucleotide. By way of another example, the examination reaction comprises one type of nucleotide and the incorporation reaction comprises four types of nucleotides, or vice versa. Optionally, the examination reaction mixture is altered or replaced by the incorporation reaction mixture. Optionally, the incorporation reaction mixture includes a catalytic metal ion (e.g., a divalent catalytic metal ion), a monovalent metal cation (e.g., potassium ions or sodium ions), glutamate ions, or a combination thereof.

**[0118]** There is flexibility in the nature of the nucleotide used in the incorporation step. For example, the at least one nucleotide can include a 3'-hydroxyl group, which can be, for example, a free 3'-hydroxyl group. Optionally, the 3' position of the at least one nucleotide molecule is modified to include a 3' terminator moiety. The 3' terminator moiety may be a reversible terminator or may be an irreversible terminator. Optionally, the reversible terminator of the at least one nucleotide molecule is replaced or removed before or after the examination step.

**[0119]** Nucleotides present in the reaction mixture but not sequestered in a ternary complex may cause multiple nucleotide insertions. A wash step can be employed prior to the chemical incorporation step to ensure only the nucleotide sequestered within a trapped ternary complex is available for incorporation during the incorporation step. Optionally, free nucleotides may be removed by enzymes such as phosphatases. The trapped polymerase complex may be a

ternary complex, a stabilized ternary complex or ternary complex involving the polymerase, primed template nucleic acid and next correct nucleotide.

**[0120]** Optionally, the nucleotide enclosed within the ternary complex of the examination step is incorporated into the 3'-end of the template nucleic acid primer during the incorporation step. For example, a stabilized ternary complex of the examination step comprises an incorporated next correct nucleotide. Optionally, the nucleotide enclosed within the ternary complex of the examination step is incorporated during the examination step, but the ternary complex does not allow for the incorporation of a subsequent nucleotide. In this instance, the ternary complex is released during an incorporation step, thereby allowing a subsequent nucleotide to become incorporated.

**[0121]** Optionally, the incorporation step involves replacing a nucleotide from the examination step (e.g., the nucleotide is an incorrect nucleotide) and incorporating another nucleotide into the 3'-end of the template nucleic acid primer. The incorporation step can involve releasing a nucleotide from within a ternary complex (e.g., the nucleotide is a modified nucleotide or nucleotide analog) and incorporating a nucleotide of a different kind into the 3'-end of the primer of the primed template nucleic acid molecule. Optionally, the released nucleotide is removed and replaced with an incorporation reaction mixture containing a next correct nucleotide. For example, the incorporated nucleotide can be a reversible terminator nucleotide, such as an unlabeled reversible terminator nucleotide that does not include a detectable fluorophore.

**[0122]** Suitable reaction conditions for incorporation may involve replacing the examination reaction mixture with an incorporation reaction mixture. Optionally, nucleotides present in the examination reaction mixture are replaced with one or more nucleotides in the incorporation reaction mixture. Optionally, the polymerase present during the examination step is replaced during the incorporation step. By this approach it is possible to employ different types of polymerase in the examination and incorporation steps. Optionally, the polymerase present during the examination step is modified during the incorporation step. Optionally, the one or more nucleotides present during the examination step are modified during the incorporation step. The reaction mixture and/or reaction conditions present during the examination step may be altered by any means during the incorporation step. These means include, but are not limited to, removing reagents, chelating reagents, diluting reagents, adding reagents, altering reaction conditions such as conductivity or pH, and any combination thereof. The reagents in the reaction mixture including any combination of

polymerase, primed template nucleic acid, and nucleotide and each may be modified during the examination step and/or incorporation step.

**[0123]** Optionally, the reaction mixture employed in the incorporation step includes competitive inhibitors, where the competitive inhibitors reduce the occurrence of multiple incorporations. In one embodiment, the competitive inhibitor is a non-incorporable nucleotide. In an embodiment, the competitive inhibitor is an aminoglycoside. The competitive inhibitor is capable of replacing either the nucleotide or the catalytic metal ion in the active site, such that after the first incorporation the competitive inhibitor occupies the active site preventing a second incorporation. In some embodiments, both an incorporable nucleotide and a competitive inhibitor are introduced in the incorporation step, such that the ratio of the incorporable nucleotide and the inhibitor can be adjusted to ensure incorporation of a single nucleotide at the 3'-end of the primer.

**[0124]** Optionally, the provided reaction mixtures including the incorporation reaction mixtures include at least one nucleotide molecule that is a non-incorporable nucleotide or a nucleotide incapable of incorporation into the nucleic acid strand. In other words, the provided reaction mixtures can include one or more nucleotide molecules incapable of incorporation into the primer of the primed template nucleic acid molecule. Such nucleotides incapable of incorporation include, for example, diphosphate nucleotides. For example, the nucleotide may contain modifications to the triphosphate group that make the nucleotide non-incorporable. Examples of non-incorporable nucleotides may be found in U.S. Pat. No. 7,482,120, which is incorporated by reference herein in its entirety. Optionally, the primer may not contain a free hydroxyl group at its 3'-end, thereby rendering the primer incapable of incorporating any nucleotide, and, thus, making any nucleotide non-incorporable.

**[0125]** A polymerase inhibitor optionally may be included with the reaction mixtures containing test nucleotides in the examination step to trap the polymerase on the nucleic acid upon binding the next correct nucleotide. Optionally, the polymerase inhibitor is a pyrophosphate analog. Optionally, the polymerase inhibitor is an allosteric inhibitor. Optionally, the polymerase inhibitor is a DNA or an RNA aptamer. Optionally, the polymerase inhibitor competes with a catalytic-ion binding site in the polymerase. Optionally, the polymerase inhibitor is a reverse transcriptase inhibitor. The polymerase inhibitor may be an HIV-1 reverse transcriptase inhibitor or an HIV-2 reverse transcriptase inhibitor. The HIV-1 reverse transcriptase inhibitor may be a (4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-one.

**[0126]** The provided method may further include preparing the primed template nucleic acid molecule for a next examination step after the incorporation step. Optionally, the preparing includes subjecting the primed template nucleic acid or the nucleic acid/polymerase complex to one or more wash steps; a temperature change; a mechanical vibration; a pH change; or an optical stimulation. Optionally, the wash step comprises contacting the primed template nucleic acid or the primed template nucleic acid/polymerase complex with one of more buffers, detergents, protein denaturants, proteases, oxidizing agents, reducing agents, or other agents capable of releasing internal crosslinks within a polymerase or crosslinks between a polymerase and nucleic acid.

**[0127]** Optionally, the method further comprises repeating the examination step and the incorporation step to sequence a template nucleic acid molecule. The examination step may be repeated one or more times prior to performing the incorporation step. Optionally, two consecutive examination steps comprise reaction mixtures with different nucleotide molecules. Optionally, the examination step involves the use of a reaction mixture that includes combinations of different nucleotides, such as pairwise combinations of nucleotides. Optionally, prior to incorporating the single nucleotide into the primed template nucleic acid molecule, the first reaction mixture is replaced with a second reaction mixture that includes a polymerase and 1, 2, 3, or 4 types of nucleotide molecules. Optionally, the nucleotide molecules are selected from dATP, dTTP (or dUTP), dCTP, and dGTP.

**[0128]** In the provided sequencing methods, the next base is identified before the incorporation step, allowing the incorporation step to not require labeled reagents and/or monitoring. Thus, in the provided methods, a nucleotide, optionally, does not contain an attached detectable tag or label. Optionally, the nucleotide contains a detectable label, but the label is not detected in the method. Optionally, the correct nucleotide does not contain a detectable label; however, an incorrect or non-complementary nucleotide to the next base contains a detectable label.

**[0129]** The examination step of the sequencing reaction may be repeated 1, 2, 3, 4 or more times prior to the incorporation step. The examination and incorporation steps may be repeated until the desired sequence of the template nucleic acid is obtained.

**[0130]** The formation of the ternary complex or the stabilized ternary complex can be employed to ensure that only one nucleotide is added to the template nucleic acid primer per cycle of sequencing, wherein the added nucleotide is sequestered within the ternary complex. The controlled incorporation of a single nucleotide per sequencing cycle enhances sequencing accuracy for nucleic acid regions comprising homopolymer repeats.

Reaction Mixtures

**[0131]** Nucleic acid sequencing reaction mixtures, or simply "reaction mixtures," typically include reagents that are commonly present in polymerase based nucleic acid synthesis reactions. Reaction mixture reagents include, but are not limited to, enzymes (e.g., polymerase), dNTPs, template nucleic acids, primer nucleic acids, salts, buffers, small molecules, co-factors, metals, and ions. The ions may be catalytic ions, divalent catalytic ions, non-catalytic ions, non-covalent metal ions, or a combination thereof. The reaction mixture can include salts, such as NaCl, KCl, potassium acetate, ammonium acetate, potassium glutamate, NH4Cl, or (NH4HSO4), that ionize in aqueous solution to yield monovalent cations. The reaction mixture can include a source of ions, such as Mg2+ or Mn2+, Co2+, Cd2+ or Ba2+ ions. The reaction mixture can include tin, Ca2+, Zn2+, Cu2+, Co2+, Fe(II)SO4, or Ni2+, or other divalent non-catalytic metal cation that stabilizes ternary complexes by inhibiting formation of phosphodiester bonds between the primed template nucleic acid molecule and the cognate nucleotide.

**[0132]** The buffer can include Tris, Tricine, HEPES, MOPS, ACES, MES, phosphate-based buffers, and acetate-based buffers. The reaction mixture can include chelating agents such as EDTA, EGTA, and the like. Optionally, the reaction mixture includes cross-linking reagents. Provided herein are first reaction mixtures, optionally, used during the examination step, as well as incorporation reaction mixtures used during nucleotide incorporation that can include one or more of the aforementioned agents. First reaction mixtures when used during examination can be referred to herein as examination reaction mixtures. Optionally, the first reaction mixture comprises a high concentration of salt; a high pH; 1, 2, 3, 4, or more types of nucleotides; potassium glutamate; a chelating agent; a polymerase inhibitor; a catalytic metal ion; a non-catalytic metal ion; or any combination thereof. The first reaction mixture can include 10 mM to 1.6 M of potassium glutamate (including any amount between 10 mM and 1.6 M). Optionally, the incorporation reaction mixture comprises a catalytic metal ion; 1, 2, 3, 4, or more types of nucleotides; potassium chloride; a non-catalytic metal ion; or any combination thereof.

**[0133]** The provided methods are conducted under reaction conditions that modulate the formation and stabilization of a ternary complex during an examination step. The reaction conditions of the examination step favor the formation and/or stabilization of a ternary complex encapsulating a nucleotide and hinder the formation and/or stabilization of a binary complex. The binary interaction between the polymerase and template nucleic acid may be manipulated by modulating sequencing reaction parameters such as ionic strength, pH,

temperature, or any combination thereof, or by the addition of a binary complex destabilizing agent to the reaction. Optionally, high salt (e.g., 50 to 1,500 mM) and/or pH changes are utilized to destabilize a binary complex. Optionally, a binary complex may form between a polymerase and a template nucleic acid during the examination or incorporation step of the sequencing reaction, regardless of the presence of a nucleotide. Optionally, the reaction conditions favor the stabilization of a ternary complex and destabilization of a binary complex. By way of example, the pH of the examination reaction mixture can be adjusted from 4.0 to 10.0 to favor the stabilization of a ternary complex and destabilization of a binary complex. Optionally, the pH of the examination reaction mixture is from 4.0 to 6.0. Optionally, the pH of the examination reaction mixture is 6.0 to 10.0.

**[0134]** The provided sequencing methods disclosed herein promote polymerase interaction with the nucleotides and template nucleic acid in a manner that reveals the identity of the next base while controlling the chemical addition of a nucleotide. Optionally, the methods are performed in the absence of detectably labeled nucleotides, or in the presence of labeled nucleotides wherein the labels are not detected.

**[0135]** Provided herein are methods for the formation and/or stabilization of a ternary complex comprising a polymerase bound to a primed template nucleic acid and a nucleotide enclosed within the polymerase-template nucleic acid complex, under examination reaction conditions. Examination reaction conditions may inhibit or attenuate nucleotide incorporation. Optionally, incorporation of the enclosed nucleotide is inhibited and the complex is stabilized or trapped in a pre-chemistry conformation or a ternary complex. Optionally, the enclosed nucleotide is incorporated and a subsequent nucleotide incorporation is inhibited. In this instance, the complex is stabilized or trapped in a pre-translocation conformation. For the sequencing reactions provided herein, the ternary complex is stabilized during the examination step, allowing for controlled nucleotide incorporation. Optionally, a stabilized ternary complex is a complex wherein incorporation of an enclosed nucleotide is attenuated, either transiently (e.g., to examine the complex and then incorporate the nucleotide) or permanently (e.g., for examination only) during an examination step. Optionally, a stabilized ternary complex allows for the incorporation of the enclosed nucleotide, but does not allow for the incorporation of a subsequent nucleotide. Optionally, the closed-complex is stabilized in order to monitor any polymerase interaction with a template nucleic acid in the presence of a nucleotide for identification of the next base in the template nucleic acid.

**[0136]** Optionally, the enclosed nucleotide has severely reduced or disabled binding to the template nucleic acid in the ternary complex. Optionally, the enclosed nucleotide is base-paired to the template nucleic acid at a next base. Optionally, the identity of the polymerase, nucleotide, primer, template nucleic acid, or any combination thereof, affects the interaction between the enclosed nucleotide and the template nucleic acid in the ternary complex.

**[0137]** Optionally, the enclosed nucleotide is bound to the polymerase of the closed-complex. Optionally, the enclosed nucleotide is weakly associated with the polymerase of the ternary complex. Optionally, the identity of the polymerase, nucleotide, primer, template nucleic acid, or any combination thereof, affects the interaction between the enclosed nucleotide and the polymerase in the ternary complex. For a given polymerase, each nucleotide has a different affinity for the polymerase than another nucleotide. Optionally, this affinity is dependent, in part, on the template nucleic acid and/or the primer.

**[0138]** The ternary complex may be transiently formed. Optionally, the enclosed nucleotide is a next correct nucleotide. In some methods, the presence of the next correct nucleotide contributes, in part, to the stabilization of a ternary complex. Optionally, the enclosed nucleotide is not a next correct nucleotide.

**[0139]** Optionally, the examination reaction condition comprises a plurality of primed template nucleic acids, polymerases, nucleotides, or any combination thereof. Optionally, the plurality of nucleotides comprises 1, 2, 3, 4, or more types of different nucleotides, for example dATP, dTTP (or dUTP), dGTP, and dCTP. Optionally, the plurality of template nucleic acids is a clonal population of template nucleic acids.

**[0140]** The examination reaction mixture can include other molecules including, but not limited to, enzymes. Optionally, the examination reaction mixture comprises any reagents or biomolecules generally present in a nucleic acid polymerization reaction. Reaction components may include, but are not limited to, salts, buffers, small molecules, detergents, crowding agents, metals, and ions. Optionally, properties of the reaction mixture may be manipulated, for example, electrically, magnetically, and/or with vibration.

Nucleotides and Nucleotide Analogs

**[0141]** Optionally, a ternary complex of an examination step comprises either a native nucleotide, or a nucleotide analog or modified nucleotide to facilitate stabilization of the ternary complex. Optionally, a nucleotide analog comprises a nitrogenous base, five-carbon sugar, and phosphate group; wherein any component of the nucleotide may be modified and/or replaced. Nucleotide analogs may be non-incorporable nucleotides. Non-incorporable

nucleotides may be modified to become incorporable at any point during the sequencing method.

**[0142]** Nucleotide analogs include, but are not limited to, alpha-phosphate modified nucleotides, alpha-beta nucleotide analogs, beta-phosphate modified nucleotides, beta-gamma nucleotide analogs, gamma-phosphate modified nucleotides, caged nucleotides, or ddNTPs. Examples of nucleotide analogs are described in U.S. Pat. No. 8,071,755, which is incorporated by reference herein in its entirety.

**[0143]** Nucleotide analogs can include terminators that reversibly prevent nucleotide incorporation at the 3'-end of the primer. One type of reversible terminator is a 3'-O-blocked reversible terminator. Here the terminator moiety is linked to the oxygen atom of the 3'-OH end of the 5-carbon sugar of a nucleotide. For example, U.S. 7,544,794 and U.S. 8,034,923 (the disclosures of these patents are incorporated by reference) describe reversible terminator dNTPs having the 3'-OH group replaced by a 3'-ONH2 group. Another type of reversible terminator is a 3'- unblocked reversible terminator, wherein the terminator moiety is linked to the nitrogenous base of a nucleotide. For example, U.S. 8,808,989 (the disclosure of which is incorporated by reference) discloses particular examples of base-modified reversible terminator nucleotides that may be used in connection with the methods described herein. Other reversible terminators that similarly can be used in connection with the methods described herein include those described in U.S. 7,956,171, U.S. 8,071,755, and U.S. 9,399,798 (the disclosures of these U.S. patents are incorporated by reference). For reviews of nucleotide analogs having terminators see e.g., Mu, R., et al., "The History and Advances of Reversible Terminators Used in New Generations of Sequencing Technology," Genomics, Proteomics & Bioinformatics 11(1):34-40 (2013). Optionally, one or more native nucleotides employed during the examination step is replaced by a second type of nucleotide that is incorporated during the incorporation step. For example, nucleotides present in the reaction mixture used during an examination step may be replaced by nucleotide analogs that include reversible terminator moieties (e.g., positioned on the base or sugar of the nucleotide molecule).

**[0144]** Optionally, nucleotide analogs have terminator moieties that irreversibly prevent nucleotide incorporation at the 3'-end of the primer. Irreversible nucleotide analogs include 2', 3'-dideoxynucleotides, ddNTPs (ddGTP, ddATP, ddTTP, ddCTP). Dideoxynucleotides lack the 3'-OH group of dNTPs that is essential for polymerase-mediated synthesis.

**[0145]** Optionally, non-incorporable nucleotides comprise a blocking moiety that inhibits or prevents the nucleotide from forming a covalent linkage to a second nucleotide (3'-OH of a

primer) during the incorporation step of a nucleic acid polymerization reaction. The blocking moiety can be removed from the nucleotide, allowing for nucleotide incorporation.

**[0146]** Optionally, a nucleotide analog present in a ternary complex renders the ternary complex stable. Optionally, the nucleotide analog is non-incorporable. Optionally, the nucleotide analog is released and a native nucleotide is incorporated. Optionally, the ternary complex is released, the nucleotide analog is modified, and the modified nucleotide analog is incorporated. Optionally, the ternary complex is released under reaction conditions that modify and/or destabilize the nucleotide analog in the ternary complex.

**[0147]** Optionally, a nucleotide analog present in a ternary complex is incorporated and the ternary complex is stabilized. The ternary complex may be stabilized by the nucleotide analog, or for example, by any stabilizing methods disclosed herein. Optionally, the nucleotide analog does not allow for the incorporation of a subsequent nucleotide. The ternary complex can be released, for example, by any methods described herein, and the nucleotide analog is modified. The modified nucleotide analog may allow for subsequent incorporation of a nucleotide to its 3'-end.

**[0148]** Optionally, a nucleotide analog is present in the reaction mixture during the examination step. For example, 1, 2, 3, 4 or more nucleotide analogs are present in the reaction mixture during the examination step. Optionally, a nucleotide analog is replaced, diluted, or sequestered during an incorporation step. Optionally, a nucleotide analog is replaced with a native nucleotide. The native nucleotide may include a next correct nucleotide. Optionally, a nucleotide analog is modified during an incorporation step. The modified nucleotide analog can be similar to or the same as a native nucleotide.

**[0149]** Optionally, a nucleotide analog has a different binding affinity for a polymerase than a native nucleotide. Optionally, a nucleotide analog has a different interaction with a next base than a native nucleotide. Nucleotide analogs and/or non-incorporable nucleotides may base-pair with a complementary base of a template nucleic acid.

**[0150]** Optionally, a nucleotide analog is a nucleotide, modified or native, fused to a polymerase. Optionally, a plurality of nucleotide analogs comprises fusions to a plurality of polymerases, wherein each nucleotide analog comprises a different polymerase.

**[0151]** A nucleotide can be modified to favor the formation of a closed-complex over the formation of a binary complex. The nucleotide modifications may be genetically engineered. A nucleotide may be selected or modified to have a high affinity for a polymerase, wherein the polymerase binds to a nucleotide prior to binding to the template nucleic acid.

**[0152]** Any nucleotide modification that traps the polymerase in a ternary complex may be used in the methods disclosed herein. The nucleotide may be trapped permanently or transiently. Optionally, the nucleotide analog is not the means by which a closed-complex is stabilized. Any ternary complex stabilization method may be combined in a reaction utilizing a nucleotide analog.

**[0153]** Optionally, a nucleotide analog that allows for the stabilization of a closed-complex is combined with reaction conditions that usually release the ternary complex. The conditions include, but are not limited to, the presence of a release reagent (e.g., catalytic metal ion, such as magnesium or manganese). Optionally, the ternary complex is stabilized even in the presence of a catalytic metal ion. Optionally, the ternary complex is released even in the presence of a nucleotide analog. Optionally, the stabilization of the closed-complex is dependent, in part, on the concentrations and/or identity of the stabilization reagent and/or release reagents, and any combination thereof. Optionally, the stabilization of a ternary complex using nucleotide analogs is combined with additional reaction conditions that function to stabilize a ternary complex, including, but not limited to, sequestering, removing, reducing, omitting, and/or chelating a catalytic metal ion; the presence of a polymerase inhibitor, cross-linking agent; and any combination thereof.

**[0154]** Optionally, one or more nucleotides can be labeled with distinguishing and/or detectable tags or labels, however such tags or labels are not detected during examination, identification of the base or incorporation of the base, and such tags or labels are not detected during the sequencing methods disclosed herein. The tags may be distinguishable by means of their differences in fluorescence, Raman spectrum, charge, mass, refractive index, luminescence, length, or any other measurable property. The tag may be attached to one or more different positions on the nucleotide, so long as the fidelity of binding to the polymerase-nucleic acid complex is sufficiently maintained to enable identification of the complementary base on the template nucleic acid correctly. Optionally, the tag is attached to the nucleobase of the nucleotide. Under suitable reaction conditions, the tagged nucleotides may be enclosed in a ternary complex with the polymerase and the primed template nucleic acid. Alternatively, a tag is attached to the gamma phosphate position of the nucleotide.

Enhancing Nucleotide Identification Using a Plurality of Nucleotides in Multiple Examination Steps

**[0155]** The disclosed sequencing-by-binding technique can be performed using more than one nucleotide during each cycle of an examination step. For example, a single examination

step optionally can be conducted using two, three, or even four different nucleotides. Optionally, each of the nucleotides is an unlabeled nucleotide, such as a native nucleotide (i.e.., dATP, dGTP, dCTP, dTTP or dUTP). Preferably, a primed template nucleic acid molecule is contacted with a plurality of reaction mixtures in a serial fashion, without incorporation of any nucleotide into the primed template nucleic acid. Optionally, each different reaction mixture includes a polymerase and a different combination of two or three different nucleotides. For example, there can be four different reaction mixtures where, in aggregate, each different nucleotide (e.g., dATP, dGTP, dCTP, and dTTP) is present two times. This could be accomplished, for example, by using the following four combinations of nucleotides: (dATP and dTTP), (dATP and dGTP), (dTTP and dCTP), and (dGTP and dCTP). An alternative would be the combinations: (dGTP and dCTP), (dGTP and dTTP), (dATP and dCTP), and (dATP and dTTP). Yet another alternative would be the combinations: (dATP and dGTP), (dATP and dCTP), (dGTP and dTTP), and (dCTP and dTTP). Examination steps can be conducted using four combinations of two different nucleotides, one after the other (i.e., such that the first combination is replaced by the second combination, the second replaced by the third, and the third replaced by the fourth). When this is the case, and when monitoring of the interaction of the polymerase with the primed template nucleic acid yields a signal confirming the binding interaction, the next correct nucleotide can be identified as the nucleotide common to two different nucleotide combinations yielding positive binding signals. If it is desired to represent each different nucleotide three times among the collection of nucleotide combinations, an exemplary combination could be: (dATP and dTTP), (dATP and dGTP), (dATP and dCTP), (dTTP and dGTP), (dTTP and dCTP), and (dGTP and dCTP). Examination steps can be conducted using six combinations of two different nucleotides, one after the other (i.e., such that the first combination is replaced by the second combination, the second replaced by the third, the third replaced by the fourth, the fourth replaced by the fifth, and the fifth replaced by the sixth). When this is the case, and when monitoring of the interaction of the polymerase with the primed template nucleic acid yields a signal confirming the binding interaction, the next correct nucleotide can be identified as the nucleotide common to three different nucleotide combinations yielding a positive binding signal.

**[0156]** One advantage underlying use of more than one nucleotide during the examination step relates to confirmatory evidence that can be used for establishing the template sequence in the sequencing-by-binding procedure. When, for one or another reason, a single particular examination step yields only a moderate signal representing the binding interaction, testing

carried out using the same nucleotide in more than one combination of nucleotides offers the opportunity for detecting the binding interaction more than once for each particular nucleotide. This enhances correct base calling by reducing the incidence of erroneously low, or false-negative results in the monitoring step.

Polymerases

**[0157]** Polymerases that may be used to carry out the disclosed techniques include naturally-occurring polymerases and any modified variations thereof, including, but not limited to, mutants, recombinants, fusions, genetic modifications, chemical modifications, synthetics, and analogs. Naturally occurring polymerases and modified variations thereof are not limited to polymerases that retain the ability to catalyze a polymerization reaction. Optionally, the naturally occurring and/or modified variations thereof retain the ability to catalyze a polymerization reaction. Optionally, the naturally-occurring and/or modified variations have special properties that enhance their ability to sequence DNA, including enhanced binding affinity to nucleic acids, reduced binding affinity to nucleic acids, enhanced catalysis rates, reduced catalysis rates, etc. Mutant polymerases include polymerases wherein one or more amino acids are replaced with other amino acids (naturally or non-naturally occurring), and insertions or deletions of one or more amino acids.

**[0158]** Modified polymerases include polymerases that contain an external tag (e.g., an exogenous detectable label), which can be used to monitor the presence and interactions of the polymerase. Optionally, intrinsic signals from the polymerase can be used to monitor their presence and interactions. Thus, the provided methods can include monitoring the interaction of the polymerase, nucleotide and template nucleic acid through detection of an intrinsic signal from the polymerase. Optionally, the intrinsic signal is a light scattering signal. For example, intrinsic signals include native fluorescence of certain amino acids such as tryptophan, wherein changes in intrinsic signals from the polymerase may indicate the formation of a ternary complex.

**[0159]** Optionally, the polymerase employed during the examination step is an unlabeled polymerase, and monitoring is performed in the absence of an exogenous detectable label associated with the polymerase. Some modified polymerases or naturally occurring polymerases, under specific reaction conditions, may incorporate only single nucleotides and may remain bound to the primer-template after the incorporation of the single nucleotide. Optionally, the thumb and finger domains of the polymerase may form transient or covalent crosslinks due to their physical proximity in the closed form of the polymerase. The

crosslinks may be formed, for example by native or engineered cysteines at suitable positions on the thumb and finger domains.

**[0160]** Optionally, the polymerase employed during the examination step includes an exogenous detectable label (e.g., a fluorescent label) chemically linked to the structure of the polymerase by a covalent bond after the polymerase has been at least partially purified using protein isolation techniques. For example, the exogenous detectable label can be chemically linked to the polymerase using a free sulfhydryl or a free amine moiety of the polymerase. This can involve chemical linkage to the polymerase through the side chain of a cysteine residue, or through the free amino group of the N-terminus. In certain preferred embodiments, a fluorescent label attached to the polymerase is useful for locating the polymerase, as may be important for determining whether or not the polymerase has localized to a spot on an array corresponding to immobilized primed template nucleic acid. The fluorescent signal need not, and preferably does not change absorption or emission characteristics as the result of binding any nucleotide. Stated differently, the signal emitted by the labeled polymerase is maintained uniformly in the presence and absence of any nucleotide being investigated as a possible next correct nucleotide.

**[0161]** The term polymerase and its variants, as used herein, also refers to fusion proteins comprising at least two portions linked to each other, for example, where one portion comprises a peptide that can catalyze the polymerization of nucleotides into a nucleic acid strand is linked to another portion that comprises a second moiety, such as, a reporter enzyme or a processivity-modifying domain. For example, T7 DNA polymerase comprises a nucleic acid polymerizing domain and a thioredoxin binding domain, wherein thioredoxin binding enhances the processivity of the polymerase. Absent the thioredoxin binding, T7 DNA polymerase is a distributive polymerase with processivity of only one to a few bases. Although DNA polymerases differ in detail, they have a similar overall shape of a hand with specific regions referred to as the fingers, the palm, and the thumb; and a similar overall structural transition, comprising the movement of the thumb and/or finger domains, during the synthesis of nucleic acids.

**[0162]** DNA polymerases include, but are not limited to, bacterial DNA polymerases, eukaryotic DNA polymerases, archaeal DNA polymerases, viral DNA polymerases and phage DNA polymerases. Bacterial DNA polymerases include E. coli DNA polymerases I, II and III, IV and V, the Klenow fragment of E. coli DNA polymerase, Clostridium stercorarium (Cst) DNA polymerase, Clostridium thermocellum (Cth) DNA polymerase and Sulfolobus solfataricus (Sso) DNA polymerase. Eukaryotic DNA polymerases include DNA

polymerases α, β, γ, δ, €, η, ζ, λ, σ, μ, and k, as well as the Revl polymerase (terminal deoxycytidyl transferase) and terminal deoxynucleotidyl transferase (TdT). Viral DNA polymerases include T4 DNA polymerase, phi-29 DNA polymerase, GA-l, phi-29-like DNA polymerases, PZA DNA polymerase, phi-15 DNA polymerase, Cpl DNA polymerase, Cp7 DNA polymerase, T7 DNA polymerase, and T4 polymerase. Other DNA polymerases include thermostable and/or thermophilic DNA polymerases such as DNA polymerases isolated from Thermus aquaticus (Taq) DNA polymerase, Thermus filiformis (Tfi) DNA polymerase, Thermococcus zilligi (Tzi) DNA polymerase, Thermus thermophilus (Tth) DNA polymerase, Thermus flavusu (Tfl) DNA polymerase, Pyrococcus woesei (Pwo) DNA polymerase, Pyrococcus furiosus (Pfu) DNA polymerase and Turbo Pfu DNA polymerase, Thermococcus litoralis (Tli) DNA polymerase, Pyrococcus sp. GB-D polymerase, Thermotoga maritima (Tma) DNA polymerase, Bacillus stearothermophilus (Bst) DNA polymerase, Pyrococcus Kodakaraensis (KOD) DNA polymerase, Pfx DNA polymerase, Thermococcus sp. JDF-3 (JDF-3) DNA polymerase, Thermococcus gorgonarius (Tgo) DNA polymerase, Thermococcus acidophilium DNA polymerase; Sulfolobus acidocaldarius DNA polymerase; Thermococcus sp. go N-7 DNA polymerase; Pyrodictium occultum DNA polymerase; Methanococcus voltae DNA polymerase; Methanococcus thermoautotrophicum DNA polymerase; Methanococcus jannaschii DNA polymerase; Desulfurococcus strain TOK DNA polymerase (D. Tok Pol); Pyrococcus abyssi DNA polymerase; Pyrococcus horikoshii DNA polymerase; Pyrococcus islandicum DNA polymerase; Thermococcus fumicolans DNA polymerase; Aeropyrum pernix DNA polymerase; and the heterodimeric DNA polymerase DP1/DP2. Engineered and modified polymerases also are useful in connection with the disclosed techniques. For example, modified versions of the extremely thermophilic marine archaea Thermococcus species 9°N (e.g., Therminator DNA polymerase from New England BioLabs Inc.; Ipswich, MA) can be used. Still other useful DNA polymerases, including the 3PDX polymerase are disclosed in U.S. 8,703,461, the disclosure of which is incorporated by reference in its entirety.

**[0163]** RNA polymerases include, but are not limited to, viral RNA polymerases such as T7 RNA polymerase, T3 polymerase, SP6 polymerase, and Kll polymerase; Eukaryotic RNA polymerases such as RNA polymerase I, RNA polymerase II, RNA polymerase III, RNA polymerase IV, and RNA polymerase V; and Archaea RNA polymerase.

**[0164]** Reverse transcriptases include, but are not limited to, HIV-1 reverse transcriptase from human immunodeficiency virus type 1 (PDB 1HMV), HIV-2 reverse transcriptase from human immunodeficiency virus type 2, M-MLV reverse transcriptase from the Moloney

murine leukemia virus, AMV reverse transcriptase from the avian myeloblastosis virus, and Telomerase reverse transcriptase that maintains the telomeres of eukaryotic chromosomes.

**[0165]** Optionally, a polymerase is tagged with a chemiluminescent tag, wherein closed-complex formation is monitored as a stable luminescence signal in the presence of the appropriate luminescence triggers. The unstable interaction of the polymerase with the template nucleic acid in the presence of an incorrect nucleotide results in a measurably weaker signal compared to the ternary complex formed in the presence of the next correct nucleotide. Additionally, an optional wash step prior to triggering luminescence can remove substantially all polymerase molecules not bound in a stable ternary complex.

**[0166]** Optionally, a polymerase is tagged with an optical scattering tag, wherein ternary complex formation is monitored as a stable optical scattering signal. The unstable interaction of the polymerase with the nucleic acid in the presence of an incorrect nucleotide results in a measurably weaker signal compared to the ternary complex formed in the presence of the next correct nucleotide.

**[0167]** Optionally, the polymerase is tagged with a plasmonic nanoparticle tag, wherein the ternary complex formation is monitored as a shift in plasmonic resonance that is different from the plasmonic resonance in the absence of the ternary complex or the presence of a ternary complex comprising an incorrect nucleotide. The change in plasmon resonance may be due to the change in local dielectric environment in the ternary complex, or it may be due to the synchronous aggregation of the plasmonic nanoparticles on a cluster of clonally amplified nucleic acid molecules or another means that affects the plasmons differently in the closed-complex configuration.

**[0168]** Optionally, the polymerase is tagged with a Raman scattering tag, wherein, the ternary complex formation is monitored as a stable Raman scattering signal. The unstable interaction of polymerase with the nucleic acid in the presence of an incorrect nucleotide results in a measurably weaker signal compared to the ternary complex formed in the presence of the next correct nucleotide.

**[0169]** Optionally, a next correct nucleotide is identified by a tag on a polymerase selected or modified to have a high affinity for nucleotides, wherein the polymerase binds to a nucleotide prior to binding to the template nucleic acid. For example, the DNA polymerase X from the African Swine Fever virus has an altered order of substrate binding, where the polymerase first binds to a nucleotide, then binds to the template nucleic acid. Optionally, a polymerase is incubated with each type of nucleotide in separate compartments, where each compartment contains a different type of nucleotide and where the polymerase is labeled differently with a

tag depending on the nucleotide with which it is incubated. In these conditions, unlabeled nucleotides are bound to differently labeled polymerases. The polymerases may be the same kind of polymerase bound to each nucleotide type or different polymerases bound to each nucleotide type. The differentially tagged polymerase-nucleotide complexes may be added simultaneously to any step of the sequencing reaction. Each polymerase-nucleotide complex binds to a template nucleic acid whose next base is complementary to the nucleotide in the polymerase-nucleotide complex. The next correct nucleotide is identified by the tag on the polymerase carrying the nucleotide. The interrogation of the next template base by the labeled polymerase-nucleotide complex may be performed under non-incorporating and/or examination conditions, where once the identity of the next template base is determined, the complex is destabilized and removed, sequestered, and/or diluted and a separate incorporation step is performed in a manner ensuring that only one nucleotide is incorporated.

**[0170]** A common method to introduce a detectable tag on a polymerase involves chemical conjugation to amines or cysteines present in the non-active regions of the polymerase. Such conjugation methods are well known in the art. As non-limiting examples, n-hydroxysuccinimide esters (NHS esters) are commonly employed to label amine groups that may be found on an enzyme. Cysteines readily react with thiols or maleimide groups, while carboxyl groups may be reacted with amines by activating them with EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride). Optionally, N-Hydroxysuccinimide (NHS) chemistry is employed at pH ranges where only the N-terminal amines are reactive (for instance, pH 7), such that only a single tag is added per polymerase.

**[0171]** Optionally, the tag attached to the polymerase is a charge tag, such that the formation of stable ternary complex can be detected by electrical means by measuring changes in local charge density around the template nucleic acids. Methods for detecting electrical charges are well known in the art, comprising methods such as field-effect transistors, dielectric spectroscopy, impedance measurements, and pH measurements, among others. Field-effect transistors include, but are not limited to, ion-sensitive field-effect transistors (ISFET), charge-modulated field-effect transistors, insulated-gate field-effect transistors, metal oxide semiconductor field-effect transistors and field-effect transistors fabricated using semiconducting single wall carbon nanotubes.

**[0172]** Optionally, a charge tag is a peptide tag having an isoelectric point below about 4 or above about 10. Optionally, a polymerase comprising a peptide tag has a total isoelectric point below about 5 or above about 9. A charge tag may be any moiety which is positively or negatively charged. The charge tag may comprise additional moieties including mass and/or

labels such as dyes. Optionally, the charge tag possesses a positive or negative charge only under certain reaction conditions such as changes in pH.

**[0173]** A polymerase optionally may be labeled with a fluorophore and/or quencher. Optionally, a nucleic acid is labeled with a fluorophore and/or quencher. Optionally, one or more nucleotides are labeled with a fluorophore and/or quencher. Exemplary fluorophores include, but are not limited to, fluorescent nanocrystals; quantum dots; d-Rhodamine acceptor dyes including dichloro[R110], dichloro[R6G], dichloro[TAMRA], dichloro[ROX] or the like; fluorescein donor dye including fluorescein, 6-FAM, or the like; Cyanine dyes such as Cy3B; Alexa dyes, SETA dyes, Atto dyes such as atto 647N which forms a FRET pair with Cy3B and the like. Fluorophores include, but are not limited to, MDCC (7-diethylamino-3-[([(2- maleimidyl)ethyl]amino)carbonyl]coumarin), TET, HEX, Cy3, TMR, ROX, Texas Red, Cy5, LC red 705 and LC red 640. Fluorophores and methods for their use including attachment to polymerases and other molecules are described in The Molecular Probes® Handbook (Life Technologies, Carlsbad Calif.) and Fluorophores Guide (Promega, Madison, WI), which are incorporated herein by reference in their entireties. Exemplary quenchers include, but are not limited to, ZEN, IBFQ, BHQ-1, BHQ-2, DDQ-I, DDQ-11, Dabcyl, Qxl quencher, Iowa Black RQ, and IRDye QC-1.

**[0174]** Optionally, a conformationally sensitive dye may be attached close to the active site of the polymerase without affecting the polymerization ability or fidelity of the polymerase; wherein a change in conformation, or a change in polar environment due to the formation of a ternary complex is reflected as a change in fluorescence or absorbance properties of the dye. Common fluorophores such as cy3 and fluorescein are known to have strong solvatochromatic response to polymerase binding and ternary complex formation, to the extent that the formation of ternary complex can be distinguished clearly from the binary polymerase-nucleic acid complex. Optionally, the ternary complex can be distinguished from binary complexes based on differences in fluorescence or absorbance signals from a conformationally sensitive dye. Optionally, a solvatochromatic dye may be employed to monitor conformational transitions; wherein the change in local polar environment induced by the conformational change can be used as the reporter signal. Solvatochromatic dyes include, but are not limited to, Reichart's dye, IR44, merocyanine dyes (e.g., merocyanine 540), 4-[2-N- substituted-1,4-hydropyridin-4-ylidine)ethylidene]cyclohexa-2,5-dien-1-one, red pyrazolone dyes, azomethine dyes, indoaniline dyes, diazamerocyanine dyes, indigoid dyes, as exemplified by indigo, and others as well as mixtures thereof. Methods to introduce dyes or fluorophores to specific sites of a polymerase are well known in the art. As a non-

limiting example, a procedure for site specific labeling of a T7 DNA polymerase with a dye is provided in Yu-Chih Tsai, Zhinan Jin, and Kenneth A. Johnson, "Site-Specific Labeling of T7 DNA Polymerase with a Conformationally Sensitive Fluorophore and Its Use in Detecting Single-Nucleotide Polymorphisms," Analytical Biochemistry 384, no. 1 (January 1, 2009): 136-44, which is incorporated herein in its entirety by reference.

**[0175]** Optionally, a polymerase is tagged with a fluorophore at a position that could sense ternary complex formation without interfering with the reaction. The polymerase may be a native or modified polymerase. Modified polymerases include those with one or more amino acid mutations, additions, and/or deletions. Optionally, one or more, but not all, cysteine amino acids are mutated to another amino acid, such as alanine. In this case, the remaining one or more cysteines are used for site-specific conjugation to a fluorophore. Alternatively, one or more amino acids are mutated to a reactive amino acid suitable for fluorophore conjugation, such as cysteines or amino acids comprising primary amines.

**[0176]** Optionally, binding between a polymerase and a template nucleic acid in the presence of a correct nucleotide may induce a decrease in fluorescence, whereas binding with an incorrect nucleotide causes an increase in fluorescence. Binding between a polymerase and a template nucleic acid in the presence of a correct nucleotide may induce an increase in fluorescence, whereas binding with an incorrect nucleotide causes a decrease in fluorescence. The fluorescent signals may be used to monitor the kinetics of a nucleotide-induced conformational change and identify the next base in the template nucleic acid sequence.

**[0177]** Optionally, the polymerase/nucleic-acid interaction may be monitored by scattering signal originating from the polymerase or tags attached to the polymerase, for instance, nanoparticle tags.

**[0178]** As discussed above, polymerases may be modified to facilitate ternary complex formation and/or stabilization during the examination step of the sequencing methods described herein. Thus, a modified polymerase may be used in the provided methods. Modifications of polymerases may include cross-linking the members within the ternary complex with cross-linkers or forming disulfide bonds within the polymerase to maintain a ternary complex.

**[0179]** Optionally, cysteine residues are positioned so that when a ternary complex is formed, the cysteines are in close proximity to form at least one disulfide bond to trap the polymerase in the closed conformation. Optionally, the finger and the thumb domain of the polymerase are engineered to contain one or more cysteines each, such that in the closed-complex, the cysteines on the opposing fingers interact, forming a disulfide bond and trapping the

polymerase in its closed conformation. Introducing cysteines to suitable positions on the polymerase so as to induce disulfide bond formation can be accomplished using methods known to those in the art of protein engineering. A reducing agent such as 2-mercaptoethanol (BME), cysteine- HCl, dithiothreitol (DTT), Tris (2-carboxyethyl) phosphine (TCEP), or any combination thereof may be used to reduce the disulfide bond and release the polymerase. Optionally, nucleotides are added sequentially, one at a time, in separate examination steps along with the cysteine modified polymerase, wherein the need for additional examination reaction conditions that favor ternary complex formation and/or stabilization is optional. Optionally, 1, 2, 3, 4 or more nucleotides are added in combination (e.g., dATP, dTTP, dCTP, and dGTP), in one examination step along with the cysteine modified polymerase, wherein the need for additional examination reaction conditions that favor ternary complex formation and/or stabilization is optional.

**[0180]** Optionally, a cysteine-modified polymerase binds to a template nucleic acid without incorporating a correct nucleotide while forming a ternary complex. While in the ternary complex, the cysteines of the polymerase are close enough in space to form at least one disulfide bond, thereby stabilizing the ternary complex. In this example, the polymerase is trapped and prevented from nucleotide incorporation.

**[0181]** Optionally, a nucleotide present in the examination reaction mixture is a next correct nucleotide, and the cysteine-modified polymerase binds to a template nucleic acid and incorporates the next correct nucleotide forming a ternary complex; wherein while in the closed-complex, the cysteines of the polymerase are close enough in space to form at least one disulfide bond, thereby stabilizing the ternary complex. After ternary complex stabilization and monitoring, an incorporation step can be performed wherein a reducing agent breaks the disulfide bond, releasing the polymerase from the ternary complex. The reducing agent may then be removed, diluted, or sequestered and another examination step may be performed.

**[0182]** Optionally, the nucleotide of the disulfide-stabilized ternary complex is incorporated prior to or during stabilization of the ternary complex. An incorporation step may be performed by reducing the disulfide bond to allow for subsequent nucleotide incorporation and/or an additional examination step.

**[0183]** Optionally, one nucleotide is added to the reaction mixture during the examination step. Optionally, 1, 2, 3, 4 or more nucleotides are added to the reaction mixture during the examination step. Optionally, the next correct nucleotide is enclosed within the ternary complex. Optionally, an incorrect nucleotide is enclosed within the ternary complex.

**[0184]** Optionally, a polymerase may form a disulfide bond with itself after formation of a ternary complex. A polymerase can form a disulfide bond to the primed template nucleic acid after formation of a ternary complex. The ternary complex may include a next correct nucleotide based-paired to the next base and/or incorporated to the primer of the primed template nucleic acid. Optionally, the ternary complex comprises an incorrect nucleotide, wherein binding to the next base and/or incorporation is attenuated.

**[0185]** Optionally, the polymerase is stabilized via cross-linking methods involving the polymerase of the ternary complex. The cross-linking methods do not need to be reversible, as the polymerase can be unbound from the nucleic acid using other means, such as enzymatic or chemical cleavage, denaturation or any combination thereof. Denaturants include, but are not limited to, acids such as acetic acid, or trichloroacetic acid; solvents such as ethanol or methanol; chaotropic agents such as urea, guanidinium chloride, lithium perchlorate; surfactants such as sodium dodecyl sulfate; or any combination thereof. Chemical cleavage includes the use of one or more of natural, modified, or commercially available proteases. Additional methods for releasing a cross-linked polymerase include, but are not limited to, altering pH, temperature, ionic strength, or any combination thereof.

**[0186]** The ternary complex is formed without incorporation of the enclosed nucleotide during an examination step of a reaction. Optionally, the examination reaction mixture at any point in time includes a cross-linking agent, wherein the ternary complex is cross-linked. In this example, the polymerase is trapped and prevented from nucleotide incorporation. Optionally, a nucleotide present in the examination reaction mixture is a next correct nucleotide, and a polymerase binds to a template nucleic acid and incorporates the next correct nucleotide forming a ternary complex; wherein, while in the ternary complex, a cross-linking agent is available to trap the ternary complex. After ternary complex stabilization and monitoring, an incorporation step can be performed wherein the ternary complex is released from its closed-conformation. Optionally, a ternary complex is formed and the next correct nucleotide is incorporated into the template nucleic acid primer. The cross-linking inhibits the polymerase from translocating to the next base position and the next nucleotide cannot be added until the polymerase is no longer cross-linked. Optionally, the nucleotide of the cross-linked-stabilized ternary complex is incorporated prior to or during stabilization of the ternary complex. An incorporation step may be performed by cleaving the linkage and/or denaturing the polymerase to allow for subsequent nucleotide incorporation and/or an additional examination step.

**[0187]** Optionally, a polymerase may be cross-linked to itself after formation of a ternary complex. Thus, a polymerase can be cross-linked to the primed template nucleic acid after formation of a ternary complex. The ternary complex may include a next correct nucleotide which is based-paired to the next base and/or incorporated to the primer of the primed template nucleic acid. Optionally, the ternary complex comprises an incorrect nucleotide who's binding to the next base and/or incorporation is attenuated.

**[0188]** Optionally, a polymerase is modified to favor the formation of a closed-complex over the formation of a binary complex. The polymerase modifications may be genetically engineered. Polymerases may be selected based on their selective binding affinities to the template nucleic acid. A polymerase may be selected or modified to have a high affinity for nucleotides, wherein the polymerase binds to a nucleotide prior to binding to the template nucleic acid. For example, the DNA polymerase X from the African Swine Fever virus has an altered order of substrate binding, where the polymerase first binds to a nucleotide, then binds to the template nucleic acid. Polymerases that bind to nucleotides first may be utilized to develop novel sequencing schemes. Polymerase modifications can be designed to trap the polymerase in a ternary complex in the methods disclosed herein. The polymerase may be trapped permanently or transiently.

**[0189]** Optionally, a modified polymerase that allows for the stabilization of a ternary complex is combined with reaction conditions, usually to release the ternary complex, including, but not limited to, the presence of a release reagent (e.g., catalytic metal ion, such as magnesium or manganese). Optionally, the ternary complex is stabilized even in the presence of a catalytic metal ion. Optionally, the ternary complex is released even in the presence of a cross-linking agent. Optionally, the stabilization of the closed- complex is dependent, in part, on the concentrations and/or identity of the stabilization reagent and/or the release reagents, and any combination thereof. Optionally, the stabilization of a ternary complex using one or more modified polymerases is combined with additional reaction conditions to stabilize a ternary complex, including, but not limited to, sequestering, removing, reducing, omitting, and/or chelating a catalytic metal ion; the presence of a polymerase inhibitor, or non-incorporable nucleotide; and any combination thereof.

Polymerase Delivery Reagents

**[0190]** In one aspect, the disclosed technique relates to a method of delivering polymerase and nucleotide to a population of immobilized nucleic acid features, where each feature includes one or more primed template nucleic acid molecules. The method includes the step

of contacting the population of immobilized nucleic acid features with a first reagent that includes: a polymerase, a first nucleotide, and at least one non-immobilized primed template nucleic acid molecule that is free in solution. The first nucleotide of the first reagent is not the next correct nucleotide for any of the non-immobilized primed template nucleic acid molecules of the first reagent. As well, the contacting step takes place under conditions that stabilize ternary complexes, and that inhibit or preclude catalysis of phosphodiester bond formation by the polymerase. By this procedure, compared to the use of reagents containing the polymerase and the first nucleotide in the absence of said non-immobilized primed template nucleic acid molecule of the first reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule to form binary complexes is reduced. In one embodiment of the technique, there is the further step of replacing the first reagent with a second reagent that includes: the same type of polymerase of the first reagent, a second nucleotide, and at least one non-immobilized primed template nucleic acid molecule that is free in solution. Here the first and second nucleotides are different from each other. The second nucleotide is not the cognate nucleotide for any of the non-immobilized primed template nucleic acid molecules of the second reagent. As well, the replacing step takes place under conditions that stabilize ternary complexes and inhibit or preclude catalysis of phosphodiester bond formation by the polymerase. When this is the case, compared to the use of reagents that include the polymerase and the second nucleotide in the absence of said non-immobilized primed template nucleic acid molecule of the second reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule to form binary complexes is reduced. Optionally, the non-immobilized primed template nucleic acid molecules of the first and second reagents are different from each other.

**[0191]** In another aspect, a reaction mixture is disclosed. The reaction mixture includes: a plurality of immobilized nucleic acid features, where each feature includes an immobilized primed template nucleic acid; a polymerase; a non-immobilized primed template nucleic acid molecule (i.e., free in solution); and a nucleotide. The nucleotide is not the cognate nucleotide for the non-immobilized primed template nucleic acid molecule. As well, nucleotide-independent binding of the polymerase to the immobilized primed template nucleic acid to form binary complexes is reduced by the presence of the non-immobilized primed template nucleic acid molecule. In one preferred embodiment, the polymerase, the immobilized primed template nucleic acid, and the nucleotide form a stabilized ternary complex that is inhibited or precluded from incorporating the nucleotide into the immobilized primed template nucleic acid.

**[0192]** In yet another aspect, there is disclosed a polymerase delivery reagent that includes: a polymerase; at least one non-immobilized primed template nucleic acid molecule; and at least one nucleotide. Here, none of the nucleotides is a next correct nucleotide for any of the non-immobilized primed template nucleic acid molecules. Optionally, the polymerase delivery reagent further includes a ternary complex stabilizing agent that inhibits phosphodiester bond formation by the solution-phase polymerase. Optionally, the at least one nucleotide includes at least one native nucleotide. Optionally, the solution-phase primed template nucleic acid molecule includes no more than three different types of primed template nucleic acid molecule, each with a different 3' terminal nucleotide. For example, the polymerase optionally is a mutant polymerase without catalytic phosphodiester bond forming activity in the presence of Mg2+ ions.

Use of Polymerase Inhibitors to Stabilize Ternary Complexes

**[0193]** A ternary complex may be formed and/or stabilized by the addition of a polymerase inhibitor to the examination reaction mixture. Inhibitor molecules phosphonoacetate, (phosphonoacetic acid) and phosphonoformate (phosphonoformic acid, common name Foscarnet), Suramin, Aminoglycosides, INDOPY-1 and Tagetitoxin are non-limiting examples of uncompetitive or noncompetitive inhibitors of polymerase activity. The binding of the inhibitor molecule, near the active site of the enzyme, traps the polymerase in either a pre-translocation or post-translocation step of the nucleotide incorporation cycle, stabilizing the polymerase in its ternary complex conformation before or after the incorporation of a nucleotide, and forcing the polymerase to be bound to the template nucleic acid until the inhibitor molecules are not available in the reaction mixture by removal, dilution or chelation.

**[0194]** Thus, provided is a method for sequencing a template nucleic acid molecule including an examination step including providing a template nucleic acid molecule primed with a primer; contacting the primed template nucleic acid molecule with a first reaction mixture comprising a polymerase, a polymerase inhibitor and at least one unlabeled nucleotide molecule; monitoring the interaction of the polymerase with the primed template nucleic acid molecule in the presence of the unlabeled nucleotide molecule without incorporation of the nucleotide into the primer of the primed template nucleic acid molecule; and identifying the nucleotide that is complementary to the next base of the primed template nucleic acid molecule by the monitored interaction. The polymerase inhibitor prevents the incorporation of the unlabeled nucleotide molecule into the primer of the primer template nucleic acid. Optionally, the inhibitor is a non-competitive inhibitor, an allosteric inhibitor, or an

uncompetitive allosteric inhibitor. Optionally, the polymerase inhibitor competes with a catalytic ion binding site in the polymerase.

**[0195]** Aminoglycosides non-competitively inhibit polymerase activity by displacing magnesium binding sites in a Klenow polymerase. The non-competitive nature of the interaction with respect to nucleotide binding allows the polymerase to interact with the template nucleic acid and nucleotide, affecting only the catalytic step of nucleotide incorporation.

**[0196]** One inhibitor molecule is the drug Efavirenz, which acts as a non-competitive inhibitor to the HIV-1 reverse transcriptase. The drug has high affinity and a low off-rate for the closed- complex configuration of the polymerase, such that, once the polymerase incorporates the next correct nucleotide, the drug binds to the polymerase, preventing the polymerase from opening its fingers to allow for binding and/or incorporation of a subsequent nucleotide. If the reaction occurs under conditions that favor ternary complex formation over the formation of a binary complex, non-specific polymerase-template nucleic acid interactions can be eliminated, wherein, the binding of the polymerase signifies the added nucleotide is complementary to the next base on the template. If the reaction occurs under examination reaction conditions, the high-affinity binding of the polymerase to the template nucleic acid containing the next correct nucleotide can be used to distinguish the ternary complex from random, non-specific interaction of polymerase with the template nucleic acid. Optionally, high-affinity polymerase binding indicates nucleotide incorporation, and a separate incorporation step is not required. Once the polymerase is bound, and the binding detected, the excess nucleotides and polymerases may be removed or sequestered from the reaction mixture. The next nucleotide may be added under examination reaction conditions and the process repeated cyclically for all nucleotide types, or in a random or predetermined order, until sequencing of a desired read-length is complete.

**[0197]** Any polymerase may be chosen and a suitable non-competitive inhibitor may be uncovered using a high-throughput screening (HTS) process. Many examples of HTS processes for polymerase inhibitors are found in the literature, wherein the specific screening criteria is for non-competitive polymerase inhibitors. As a general concept, these inhibitors can be screened to have a binding site that is only exposed when the polymerase is in its closed conformation, and they bind with high affinities and very low off-rates, such that the binding of the inhibitor stabilizes the polymerase in the closed conformation. Such an inhibitor allows incorporation of a single base, after which the binding of the inhibitor prevents the polymerase from opening up to receive another nucleotide. The entire system

can be washed away, including the polymerase, before initiating the next step (examination or incorporation) in the sequencing reaction.

**[0198]** Optionally, polymerase inhibitors found to be effective in inhibiting a HIV-1 reverse transcriptase polymerase are employed to stabilize a ternary complex. Optionally, the inhibitor is an inhibitor of HIV-2 reverse transcriptase. HIV-1 reverse transcriptase inhibitors include nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). NRTIs include, but are not limited to, COMBIVIR (lamivudine and zidovudine; GlaxoSmithKline, Middlesex, UK), EMTRIVA (emtricitabine; Gilead Sciences, Foster City, CA), EPIVIR (lamivudine; GlaxoSmithKline, Middlesex, UK), EPZICOM (abacavir sulfate and lamivudine; GlaxoSmithKline, Middlesex, UK), HIVID (zalcitabine; Hoffmann-La Roche, Nutley, N.J.), RETROVIR (zidovudine; GlaxoSmithKline, Middlesex, UK), TRIZIVIR (abacavir sulfate, zidovudine, lamivudine; GlaxoSmithKline, Middlesex, UK), TRUVADA (emtricitabine/tenofovir disoproxil fumarate; Gilead Sciences, Foster City, CA), VIDEX EC (enteric coated didanosine; Bristol Myers-Squibb, New York, N.Y.), VIDEX (didanosine; Bristol Myers-Squibb, New York, N.Y.), VIREAD (tenofovir disoproxil fumarate; Gilead Sciences, Foster City, CA), ZERIT (stavudine; Bristol Myers-Squibb, New York, N.Y.), and ZIAGEN (abacavir sulfate; GlaxoSmithKline, Middlesex, UK). Examples of NNRTI include, but are not limited to, VIRAMUNE (nevirapine; Boehringer Ingelheim, Rhein, Germany), SUSTIVA (efavirenz, Bristol Myers-Squibb, New York, N.Y.), DELAVIRDINE (Rescriptor; Pfizer, New York, N.Y.), and INTELENCE (etravirine; Tibotec Therapeutics, Eastgate Village, Ireland). Optionally, NNRTIs are non-competitive polymerase inhibitors that bind to an allosteric center located near the RNA polymerase active site on subunit p66.

**[0199]** Optionally, an HIV-1 reverse transcriptase polymerase inhibitor is a (4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-one. Table 1 includes a list of 19 (4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-ones inhibitors (adapted from E. Pitta et. al., Synthesis and HIV-1 RT inhibitory action of novel (4/6-substituted benzo[d]thiazol-2-yl)thiazolidin-4-ones. Divergence from the non-competitive inhibition mechanism, Journal of Enzyme Inhibition and Medicinal Chemistry, February 2013, Vol. 28, No.1, Pages 113-122). The (4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-ones inhibitors have the following formula:

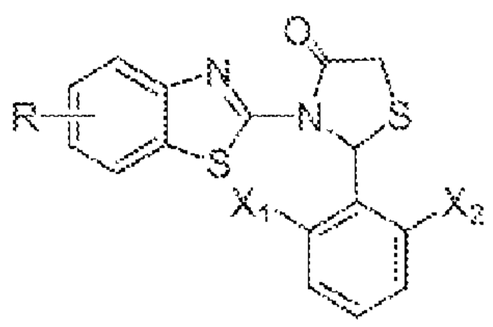


**Table 1**
(4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-ones inhibitors

| **NNRTI Inhibitor** | **R** | **$X_1$** | **$X_2$** |
|---|---|---|---|
| 1 | 4-H, 6-H | F | F |
| 2 | 4-H, 6-H | F | Cl |
| 3 | 4-H, 6-Cl | Cl | Cl |
| 4 | 4-H, 6-Cl | F | Cl |
| 5 | 4-H, 6-Cl | F | F |
| 6 | 4-H, 6-H | Cl | Cl |
| 7 | 4-H, 6-H | F | Cl |
| 8 | 4-H, 6-H | F | F |
| 9 | 4-H, 6-F | Cl | Cl |
| 10 | 4-H, 6-F | F | Cl |
| 11 | 4-H, 6-F | F | F |
| 12 | 4-H, 6-MeO | Cl | Cl |
| 13 | 4-H, 6-MeO | F | Cl |
| 14 | 4-H, 6-MeO | F | F |
| 15 | 4-MeO, 6-H | Cl | Cl |
| 16 | 4-MeO, 6-H | F | Cl |
| 17 | 4-H, 6-EtO | Cl | Cl |
| 18 | 4-H, 6-EtO | F | Cl |
| 19 | 4-H, 6-EtO | F | F |

**[0200]** Any suitable combination of polymerase inhibitors and polymerase mutants may be used so long as they trap/stabilize the ternary complex and, optionally, prevent multiple nucleotide incorporations per cycle.

**[0201]** The provided reaction mixtures can include from 100 nM to 1 mM of the polymerase inhibitor, or any amount of inhibitor between 100 nM and 1 mM. Optionally, the provided reaction mixtures can comprise from 1 to 200 μM of the polymerase inhibitor or any amount in between. Optionally, the reaction mixtures include from 30 to 150 μM of the polymerase inhibitor. Optionally, the reaction mixtures include from 30 to 70 μM of the polymerase inhibitor. Optionally, the reaction mixtures include from 60 to 140 μM of the polymerase inhibitor.

**[0202]** Optionally, the polymerase of the ternary complex is prevented from opening its finger domains and translocating to the next template nucleic acid position by using pyrophosphate analogs or other related molecules. Pyrophosphate analogs configure the polymerase in ternary complex by occupying sites close to the triphosphate binding site in the active pocket of the polymerase. Release of the pyrophosphate (PPi) is critical for the polymerase to assume the open conformation, translocate to the next template nucleic acid position, and accept the next nucleotide. The non-competitive inhibitor, such as Foscarnet (phosphonoformate), phosphonoacetate or other pyrophosphate analogs, traps the polymerase in its fingers-closed confirmation. Optionally, binding of the PPi analog is reversible, with the polymerase activity fully restored by washing away, diluting, or sequestering the inhibitor in the reaction mixture. Broadly, any non-competitive inhibitor of polymerase activity may be used during the sequencing reaction.

**[0203]** Optionally, a polymerase inhibitor which stabilizes a ternary complex is combined with reaction conditions which usually release the ternary complex, including, but not limited to, the presence of a catalytic metal ion, such as magnesium or manganese. Optionally, the ternary complex is stabilized even in the presence of a catalytic metal ion. Optionally, the ternary complex is released even in the presence of a polymerase inhibitor. Optionally, the stabilization of the ternary complex is dependent, in part, on the concentrations, the identity of the stabilization reagent, the identity of release reagents, and any combination thereof. Optionally, the stabilization of a ternary complex using polymerase inhibitors is combined with additional reaction conditions which also function to stabilize a ternary complex, including, but not limited to, sequestering, removing, reducing, omitting, and/or chelating a catalytic metal ion; the presence of a modified polymerase in the ternary complex; a non-incorporable nucleotide in the ternary complex; and any combination thereof.

Conditions for Forming and Manipulating Ternary Complexes

**[0204]** As used herein, a ternary complex includes a polymerase, a primed template nucleic acid, and a nucleotide. The ternary complex may be in a pre-chemistry conformation, wherein a nucleotide is sequestered but not incorporated. Optionally, the ternary complex may be stabilized by the presence of a chemical block on the 3' nucleotide of the primer of the primed template nucleic acid molecule (e.g., a reversible terminator moiety on the base or sugar of the nucleotide). The ternary complex may be in a pre-translocation conformation, wherein a nucleotide is incorporated by formation of a phosphodiester bond with the 3'-end of the primer in the primed template nucleic acid. The ternary complex may be formed in the

absence of catalytic metal ions, or under deficient levels of catalytic metal ions, thereby physically sequestering the next correct nucleotide within the polymerase active site without chemical incorporation. Optionally, the sequestered nucleotide may be a non-incorporable nucleotide. The ternary complex may be formed in the presence of catalytic metal ions, where the ternary complex comprises a nucleotide analog which is incorporated, but a PPi is not capable of release. In this instance, the ternary complex is stabilized in a pre-translocation conformation. Optionally, a pre-translocation conformation is stabilized by chemically cross-linking the polymerase. Optionally, the ternary complex may be stabilized by external means. In some instances, the ternary complex may be stabilized by allosteric binding of small molecules. Optionally, ternary complex may be stabilized by pyrophosphate analogs, including, but not limited to, phosphonoformates, that bind close to the active site with high affinity, preventing translocation of the polymerase.

**[0205]** As used herein, a stabilized ternary complex refers to a polymerase trapped at the polymerization initiation site (3'-end of the primer) of the primed template nucleic acid by one or a combinations of means, including but not limited to, crosslinking the thumb and finger domains in the closed conformation, binding of an allosteric inhibitor that prevents return of the polymerase to an open conformation, binding of pyrophosphate analogs that trap polymerase in the pre-translocation step, absence of catalytic metal ions in the active site of the polymerase, and addition of a non-catalytic metal ion such as nickel, barium, tin and strontium as substitutes for a catalytic metal ion. As such, the polymerase may be trapped at the polymerization initiation site even after the incorporation of a nucleotide. Therefore, the polymerase may be trapped in the pre-chemistry conformation, pre-translocation step, post-translocation step, or any intermediate step thereof, thereby allowing for sufficient examination and identification of the next correct nucleotide or base.

**[0206]** As described herein, a polymerase-based, sequencing-by-binding reaction generally involves providing a primed template nucleic acid, providing the primed template nucleic acid with a polymerase and one or more types of nucleotides, wherein the nucleotides may or may not be complementary to the next base of the primed template nucleic acid, and examining the interaction of the polymerase with the primed template nucleic acid under conditions wherein either chemical incorporation of a nucleotide to the primed template nucleic acid is disabled or severely inhibited in the pre-chemistry conformation or one or more complementary nucleotide incorporation occurs at the 3'-end of the primer. Optionally, when the pre-chemistry conformation is stabilized prior to nucleotide incorporation, preferably using stabilizers or a 3' reversibly terminated primer, a separate incorporation step

may follow the examination step to incorporate a single nucleotide at the 3'-end of the primer. Optionally, where a single nucleotide incorporation occurs, the pre-translocation conformation may be stabilized to facilitate examination and/or prevent subsequent nucleotide incorporation.

**[0207]** While single template nucleic acid molecules may be described for ease of exposition, the sequencing methods provided herein readily encompass a plurality of template nucleic acids, wherein the plurality of nucleic acids may be clonally amplified copies of a single nucleic acid, or disparate nucleic acids, including combinations, such as populations of disparate nucleic acids that are clonally amplified.

**[0208]** Optionally, the ternary complex is transiently formed during the examination step of the sequencing methods provided herein. Optionally, the ternary complex is stabilized during the examination step. The stabilized ternary complex may not allow for the incorporation of a nucleotide in a polymerization reaction during the examination step, this includes incorporation of the enclosed nucleotide and/or incorporation of a subsequent nucleotide after the enclosed nucleotide. Reaction conditions that modulate the stability of a ternary complex include, but are not limited to, the availability of catalytic metal ions, suboptimal or inhibitory metal ions, ionic strength, pH, temperature, polymerase inhibitors, cross-linking reagents, and any combination thereof. Reaction reagents modulate the stability of a ternary complex include, but are not limited to, non-incorporable nucleotides, incorrect nucleotides, nucleotide analogs, modified polymerases, template nucleic acids with non-extendible polymerization initiation sites, and any combination thereof.

**[0209]** Optionally, a ternary complex is released from its trapped or stabilized conformation, which may allow for nucleotide incorporation at the 3'-end of the primer in the primer-template nucleic acid duplex. The ternary complex can be destabilized and/or released by modulating the reaction conditions. In addition, the ternary complex can be destabilized by electrical, magnetic, and/or mechanical means. Mechanical means include mechanical agitation, for example, by using ultrasound agitation. Mechanical vibration destabilizes the closed-complex and suppresses binding of the polymerase to the DNA. Thus, rather than a wash step where the examination reaction mixture is replaced with an incorporation mixture, mechanical agitation may be used to remove the polymerase from the template nucleic acid, enabling cycling through successive incorporation steps with a single nucleotide addition per step.

**[0210]** Any combination of ternary complex stabilization or ternary complex release reaction conditions and/or methods may be combined. For example, a polymerase inhibitor which

stabilizes a ternary complex may be present in the examination reaction with a catalytic ion, which functions to release the ternary complex. In the aforementioned example, the closed-complex may be stabilized or released, depending on the polymerase inhibitor properties and concentration, the concentration of the catalytic metal ion, other reagents and/or conditions of the reaction mixture, and any combination thereof.

**[0211]** The ternary complex can be stabilized under reaction conditions where covalent attachment of a nucleotide to the 3'-end of the primer in the primed template nucleic acid is attenuated. Optionally, the ternary complex is in a pre-chemistry conformation. Optionally, the ternary complex is in a pre-translocation conformation. The formation of this ternary complex can be initiated and/or stabilized by modulating the availability of a catalytic metal ion that permits ternary complex release and/or chemical incorporation of a nucleotide to the primer in the reaction mixture. Exemplary metal ions include, but are not limited to, magnesium, manganese, cobalt, and barium. Catalytic ions (e.g., divalent catalytic metal ions) may arise from any formulation, for example, salts such as MgCl2, Mg (C2H3O2)2, and MnCl2.

**[0212]** The selection and/or concentration of the catalytic metal ion may be based on the polymerase and/or nucleotides in the sequencing reaction. For example, the HIV reverse transcriptase utilizes magnesium for nucleotide incorporation (N Kaushik 1996 Biochemistry 35:11536-11546, and H P Patel 1995 Biochemistry 34:5351-5363, which are incorporated by reference herein in their entireties). The rate of ternary complex formation using magnesium versus manganese can be different depending on the polymerase and the identity of the nucleotide. Thus, the stability of the closed-complex may differ depending on catalytic metal ion, polymerase, and/or nucleotide identity. Further, the concentration of catalytic ion necessary for ternary complex stabilization may vary depending on the catalytic metal ion, polymerase, and/or nucleotide identity and can be readily determined using the guidance provided herein. For example, nucleotide incorporation may occur at high catalytic ion concentrations of one metal ion but does not occur at low concentrations of the same metal ion, or vice versa. Therefore, modifying metal ion identity, metal ion concentration, polymerase identity, and/or nucleotide identity allows for controlled examination reaction conditions.

**[0213]** The ternary complex may be formed and/or stabilized by sequestering, removing, reducing, omitting, and/or chelating a catalytic metal ion during the examination step of the sequencing reaction so that ternary complex release and/or chemical incorporation does not occur. Chelation includes any procedure that renders the catalytic metal ion unavailable for

nucleotide incorporation, including using EDTA and/or EGTA. A reduction includes diluting the concentration of a catalytic metal ion in the reaction mixture.

**[0214]** The reaction mixture used in the examination step can be diluted or replaced with a solution comprising a non-catalytic ion, which permits ternary complex formation, but inhibits nucleotide incorporation. Optionally, a non-catalytic metal ion and a catalytic metal ion are both present in the reaction mixture, wherein one ion is present in a higher effective concentration than the other. In the provided methods, a non-catalytic ion such as cobalt can become catalytic, i.e., facilitate nucleotide incorporation at high concentrations.

**[0215]** Non-catalytic ions may be added to, or included in, a reaction mixture used under examination conditions. The reaction may already include nucleotides. Optionally, non-catalytic ions are complexed to one or more nucleotides and complexed nucleotides are added to the reaction mixture. Non-catalytic ions can complex to nucleotides by mixing nucleotides with non-catalytic ions at elevated temperatures (about 80 °C). For example, a chromium nucleotide complex may be added to a mixture to facilitate ternary complex formation and stabilization. Optionally, a chromium nucleotide complex is a chromium monodentate, bidentate, or tridentate complex. Optionally, a chromium nucleotide complex is an α-monodentate, or β-γ-bidentate nucleotide.

**[0216]** Optionally, a ternary complex is formed between a polymerase, primed template nucleic acid, and nucleotide in reaction conditions comprising Sr2+ wherein Sr2+ induces the formation of the ternary complex. The presence of Sr2+ can allow for the favorable formation of a ternary complex comprising a next correct nucleotide over the formation a complex comprising an incorrect nucleotide. Sr2+ may be present at concentrations from about 0.01 mM to about 30 mM. Optionally, Sr2+ is present as 10 mM SrCl2. The formation of the ternary complex is monitored under examination conditions to identify the next base in the template nucleic acid of the ternary complex. The affinity of the polymerase (e.g., Klenow fragment of E. coli DNA polymerase I, Bst) for each dNTPs (e.g., dATP, dTTP, dCTP, dGTP) in the presence of Sr2+ can be different. Therefore, examination can involve measuring the binding affinities of polymerase-template nucleic acids to dNTPs; wherein binding affinity is indicative of the next base in the template nucleic acid. Optionally, the binding interaction may be performed under conditions that destabilize the binary interactions between the polymerase and primed template nucleic acid. Optionally, the binding interaction may be performed under conditions that stabilize the ternary interactions between the polymerase, the primed template nucleic acid, and the next correct nucleotide. Optionally, after examination, a wash step removes unbound nucleotides, and Mg2+ is added

to the reaction to induce nucleotide incorporation and pyrophosphate (PPi) release. Optionally, the wash step comprises Sr2+ to maintain the stability of the ternary complex, preventing the dissociation of the ternary complex. The reaction may be repeated until a desired sequence read-length is obtained.

**[0217]** Optionally, a ternary complex is formed between a polymerase, primed template nucleic acid, and nucleotide in reaction conditions comprising Ni2+ wherein Ni2+ induces the formation of the ternary complex. The presence of Ni2+ can allow for the favorable formation of a ternary complex comprising a next correct nucleotide over the formation a complex comprising an incorrect nucleotide. Ni2+ may be present at concentrations from about 0.01 mM to about 30 mM. Optionally, Ni2+ is present as 10 mM NiCl2. The formation of the ternary complex is monitored under examination conditions to identify the next base in the template nucleic acid of the ternary complex. The affinity of the polymerase (e.g., Klenow fragment of E. coli DNA polymerase I, Bst) for each dNTPs (e.g., dATP, dTTP, dCTP, dGTP) in the presence of Sr2+ can be different. Therefore, examination can involve measuring the binding affinities of polymerase-template nucleic acids to dNTPs; wherein binding affinity is indicative of the next base in the template nucleic acid. Optionally, the binding interaction may be performed under conditions that destabilize the binary interactions between the polymerase and primed template nucleic acid. Optionally, the binding interaction may be performed under conditions that stabilizes the ternary interactions between the polymerase, the primed template nucleic acid, and the next correct nucleotide. Optionally, after examination, a wash removes unbound nucleotides and polymerase, and Mg2+ is added to the reaction to induce nucleotide incorporation and pyrophosphate release. Optionally, the wash buffer comprises Ni2+ to maintain the stability of the ternary complex, preventing the dissociation of the ternary complex. The reaction may be repeated until a desired sequence read-length is obtained.

**[0218]** Optionally, a ternary complex is formed between a polymerase, primed template nucleic acid, and nucleotide in reaction conditions comprising non-catalytic concentrations of Co2+ wherein Co2+ induces the formation of the ternary complex. The presence of non-catalytic concentrations of Co2+ can allow for the favorable formation of a ternary complex comprising a next correct nucleotide over the formation a complex comprising an incorrect nucleotide. Co2+ may be present at concentrations from about 0.01 mM to about 0.5 mM. Optionally, Co2+ is present as 0.5 mM CoCl2. The formation of the ternary complex is monitored under examination conditions to identify the next base in the template nucleic acid of the ternary complex. The affinity of the polymerase (e.g., Klenow fragment of E. coli

DNA polymerase I, Bst) for each dNTPs (e.g., dATP, dTTP, dCTP, dGTP) in the presence of Co2+ can be different. Therefore, examination can involve measuring the binding affinities of polymerase-template nucleic acids to dNTPs; wherein binding affinity is indicative of the next base in the template nucleic acid. Optionally, the binding interaction may be performed under conditions that destabilize the binary interactions between the polymerase and primed template nucleic acid. Optionally, the binding interaction may be performed under conditions that stabilizes the ternary interactions between the polymerase, the primed template nucleic acid, and the next correct nucleotide. After examination, a wash removes unbound nucleotides and polymerase, and Co2+ at a catalytic concentration is added to the reaction to induce nucleotide incorporation and pyrophosphate release. Optionally, the wash buffer comprises non-catalytic amounts of Co2+ to maintain the stability of the ternary complex, preventing the dissociation of the ternary complex. The reaction may be repeated until a desired sequence read-length is obtained.

**[0219]** Optionally, a catalytic metal ion (e.g., a divalent catalytic metal ion) may facilitate the formation of a closed-complex without subsequent nucleotide incorporation and ternary complex release. Optionally, a concentration of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 μM Mg2+ in a reaction mixture can induce conformational change of a polymerase to form a ternary complex without subsequent nucleotide incorporation, PPi and ternary complex release. Optionally, the concentration of Mg2+ is from about 0.5 μM to about 10 μM, from about 0.5 μM to about 5 μM, from about 0.5 μM to about 4 μM, from about 0.5 μM to about 3 μM, from about μM to about 5 μM, from about 1 μM to about 4 μM, and from about 1 μM to about 3 μM.

**[0220]** Optionally, the concentration of catalytic metal ion (e.g., a divalent catalytic metal ion) in the sequencing reaction which is necessary to allow nucleotide incorporation is from about 0.001 mM to about 10 mM, from about 0.01 mM to about 5 mM, from about 0.01 mM to about 3 mM, from about 0.01 to about 2 mM, from about 0.01 mM to about 1 mM, from about 0.05 to about 10 mM, from about 0.05 mM to about 5 mM, from about 0.05 mM to about 3 mM, from about 0.05 to about 2 mM, or from about 0.05 mM to about 1 mM. Optionally, the concentration of catalytic metal ion is from 5 to 50 mM. Optionally, the concentration of catalytic metal ion is from 5 to 15 mM, or about 10 mM.

**[0221]** A non-catalytic ion (e.g., a divalent non-catalytic metal ion) may be added to the reaction mixture at any stage including before, during, or after any of the following reaction steps: providing a primed template nucleic acid, providing a polymerase, formation of a binary complex, providing a nucleotide, formation of a pre-chemistry ternary complex,

nucleotide incorporation, formation of a pre-translocation ternary complex, and formation of a post-translocation conformation. The non-catalytic ion may be added to the reaction mixture during wash steps. The non-catalytic ion may be present through the reaction in the reaction mixture. For example, a catalytic ion is added to the reaction mixture at concentrations which dilute the non-catalytic metal ion, allowing for nucleotide incorporation.

**[0222]** The ability of catalytic and non-catalytic ions to modulate nucleotide incorporation may depend on conditions in the reaction mixture including, but not limited to, pH, ionic strength, chelating agents, chemical cross-linking, modified polymerases, non-incorporable nucleotides, mechanical or vibration energy, and electric fields.

**[0223]** Optionally, the concentration of non-catalytic metal ion (e.g., a divalent non-catalytic metal ion) in the sequencing reaction necessary to allow for ternary complex formation without nucleotide incorporation is from about 0.1 mM to about 50 mM, from about 0.1 mM to about 40 mM, from about 0.1 mM to about 30 mM, from about 0.1 mM to about 20 mM, from about 0.1 mM to about 10 mM, from about 0.1 mM to about 5 mM, from about 0.1 to about 1 mM, from about 1 mM to about 50 mM, from about 1 to about 40 mM, from about 1 mM to about 30 mM, from about 1 mM to about 20 mM, from about 1 mM to about 10 mM, from about 1 mM to about 5 mM, from about 2 mM to about 30 mM, from about 2 mM to about 20 mM, from about 2 mM to about 10 mM, or any concentration within these ranges. Non-catalytic ions useful in this regard include, but are not limited to: calcium, strontium, scandium, titanium, vanadium, chromium, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, rhodium, europium, and terbium ions. Optionally, Ni2+ is provided in an examination reaction to facilitate ternary complex formation. Optionally, Sr2+ is provided in an examination reaction to facilitate ternary complex formation. In certain embodiments, one or more non-catalytic ions may be included at an above-indicated concentration, while other non-catalytic ions are excluded from the reaction mixture during the examination step. For example, nickle or strontium may be included, while calcium is excluded.

<u>Detection Platforms: Instrumentation for Detecting the Ternary Complex</u>

**[0224]** Interaction between the polymerase and the template nucleic acid in the presence of nucleotides can be monitored with or without the use of a tagged label. For example, the sequencing reaction may be monitored by detecting the change in refractive index, charge detection, Raman scattering detection, ellipsometry detection, pH detection, size detection,

mass detection, surface plasmon resonance, guided mode resonance, nanopore optical interferometry, whispering gallery mode resonance, nanoparticle scattering, photonic crystal, quartz crystal microbalance, bio-layer interferometry, vibrational detection, pressure detection and other label free detection schemes that detect the added mass or refractive index due to polymerase binding in a ternary complex with a template nucleic acid.

**[0225]** Optionally, a Raman signature of a polymerase is exploited to detect polymerase conformational changes that occur during one or more steps of a sequencing method disclosed herein, for example ternary complex formation or release. Optionally, during one or more steps of a nucleic acid sequencing method described herein, light is directed to the sequencing reaction mixture in the visible, near infrared, or near ultraviolet range. The light interacts with molecular vibrations or other excitations in the reaction, resulting in the energy of the light being shifted. The shift in energy provides information about the vibrational modes of the reaction and therefore provides information on the configurations of reaction components (e.g., polymerase). The sequencing methods of this disclosure may be detected with surface enhanced Raman, resonance Raman, tip-enhanced Raman, polarized Raman, stimulated Raman, transmission Raman, spatially offset Raman, and hyper Raman spectroscopy.

**[0226]** Optionally, detecting a change in refractive index is accomplished in one or a combination of means, including, but not limited to, surface plasmon resonance sensing, localized plasmon resonance sensing, plasmon-photon coupling sensing, transmission sensing through sub-wavelength nanoholes (enhanced optical transmission), photonic crystal sensing, interferometry sensing, refraction sensing, guided mode resonance sensing, ring resonator sensing, or ellipsometry sensing. Optionally, nucleic acid molecules may be localized to a surface, wherein the interaction of polymerase with nucleic acids in the presence of various nucleotides may be measured as a change in the local refractive index.

**[0227]** Optionally, the template nucleic acid is tethered to or localized appropriately on or near a surface, such that the interaction of polymerase and template nucleic acid in the presence of nucleotides changes the light transmitted across or reflected from the surface. The surface may contain nanostructures. Optionally, the surface is capable of sustaining plasmons or plasmon resonance. Optionally, the surface is a photonic substrate, not limited to a resonant cavity, resonant ring or photonic crystal slab. Optionally, the surface is a guided mode resonance sensor. Optionally, the nucleic acid is tethered to, or localized appropriately on or near a nanohole array, a nanoparticle or a microparticle, such that the interaction of polymerase and template nucleic acid in the presence of nucleotides changes

the absorbance, scattering, reflection or resonance of the light interacting with the microparticle or nanoparticle.

**[0228]** Optionally, a nanohole array on a gold surface is used as a refractive index sensor. The template nucleic acid may be attached to a metal surface by standard thiol chemistry, incorporating the thiol group on one of the primers used in a PCR reaction to amplify the DNA. When the dimensions of the nanohole array are appropriately tuned to the incident light, binding of the polymerase to the template nucleic acid in the presence of nucleotides can be monitored as a change in light transmitted across the nanoholes. For both the labeled and label-free schemes, simple and straightforward measurement of equilibrium signal intensity may reveal the formation of a stable closed-complex.

**[0229]** Optionally, nucleic acid molecules are localized to a surface capable of sustaining surface plasmons, wherein the change in refractive index caused by the polymerase interaction with localized nucleic acids may be monitored through the change in the properties of the surface plasmons, wherein further, said properties of surface plasmons may include surface plasmon resonance. Surface plasmons, localized surface plasmons (LSP), or surface plasmon polaritons (SPP), arise from the coupling of electromagnetic waves to plasma oscillations of surface charges. LSPs are confined to nanoparticle surfaces, while SPPs and are confined to high electron density surfaces, at the interface between high electron mobility surfaces and dielectric media. Surface plasmons may propagate along the direction of the interface, whereas they penetrate into the dielectric medium only in an evanescent fashion. Surface plasmon resonance conditions are established when the frequency of incident electromagnetic radiation matches the natural frequency of oscillation of the surface electrons. Changes in dielectric properties at the interface, for instance due to binding or molecular crowding, affects the oscillation of surface electrons, thereby altering the surface plasmon resonance wavelength. Surfaces capable of surface plasmon resonance include, in a non-limiting manner, nanoparticles, clusters and aggregates of nanoparticles, continuous planar surfaces, nanostructured surfaces, and microstructured surfaces. Materials such as gold, silver, aluminum, high conductivity metal oxides (e.g., indium tin oxide, zinc oxide, tungsten oxide) are capable of supporting surface plasmon resonance at their surfaces.

**[0230]** Optionally, a single nucleic acid molecule, or multiple clonal copies of a nucleic acid, are attached to a nanoparticle, such that binding of polymerase to the nucleic acid causes a shift in the localized surface plasmon resonance (LSPR). Light incident on the nanoparticles induces the conduction electrons in them to oscillate collectively with a resonant frequency that depends on the nanoparticles' size, shape and composition. Nanoparticles of interest may

assume different shapes, including spherical nanoparticles, nanorods, nanopyramids, nanodiamonds, and nanodiscs. As a result of these LSPR modes, the nanoparticles absorb and scatter light so intensely that single nanoparticles are easily observed by eye using dark-field (optical scattering) microscopy. For example, a single 80-nm silver nanosphere scatters 445-nm blue light with a scattering cross-section of (3 X 10-2) m2, a million fold greater than the fluorescence cross-section of a fluorescein molecule, and a thousand fold greater than the cross-section of a similarly sized nanosphere filled with fluorescein to the self-quenching limit. Optionally, the nanoparticles are plasmon-resonant particles configured as ultra-bright, nanosized optical scatters with a scattering peak anywhere in the visible spectrum. Plasmon-resonant particles are advantageous as they do not bleach. Optionally, plasmon-resonant particles are prepared, coated with template nucleic acids, and provided in a reaction mixture comprising a polymerase and one or more nucleotides, wherein a polymerase-template nucleic acid-particle interaction is detected. One or more of the aforementioned steps may be based on or derived from one or more methods disclosed in Sheldon Schultz et al., "Single-Target Molecule Detection with Nonbleaching Multicolor Optical Immunolabels," Proceedings of the National Academy of Sciences 97, no. 3 (February 1, 2000): 996-1001, which is incorporated by reference herein in its entirety.

**[0231]** The very large extinction coefficients at resonant wavelength enables noble-metal nanoparticles to serve as extremely intense labels for near-surface interactions. Optionally, polymerase interaction with nanoparticle-localized DNA results in a shift in the resonant wavelength. The change in resonant wavelength due to binding or binding interactions can be measured in one of many ways. Optionally, the illumination is scanned through a range of wavelengths to identify the wavelength at which maximum scattering is observed at an imaging device. Optionally, broadband illumination is utilized in conjunction with a dispersive element near the imaging device, such that the resonant peak is identified spectroscopically. Optionally, the nanoparticle system may be illuminated at its resonant wavelength, or near its resonant wavelength, and any binding interactions may be observed as a drop in intensity of light scattered as the new resonant wavelength shifts away from the illumination wavelength. Depending on the positioning of the illuminating wavelength, interactions may even appear as an increase in nanoparticle scattering as the resonance peak shifts towards the illumination wavelength. Optionally, DNA-attached-nanoparticles may be localized to a surface, or, alternatively, the DNA-attached-nanoparticles may be suspended in solution. A comprehensive review of biosensing using nanoparticles is described in Jeffrey N.

Anker et al., "Biosensing with Plasmonic Nanosensors," Nature Materials 7, no. 6 (June 2008): 442-53, which is incorporated in its entirety herein by reference.

**[0232]** Optionally, nano-features capable of LSPR are lithographically patterned on a planar substrate. The two dimensional patterning of nano-features has advantages in multiplexing and high-throughput analysis of a large number of different nucleic acid molecules. Optionally, gold nanoposts are substrates for surface plasmon resonance imaging detection of polymerase-template nucleic acid interactions, wherein the nucleic acids are attached to the nanoposts. Nanostructure size and period can influence surface plasmon resonance signal enhancement, optionally, providing a 2, 3, 4, 5, 6, 7, 8-fold or higher signal amplification when compared to control films.

**[0233]** Optionally, surface plasmon resonance may be sustained in planar surfaces. A number of commercial instruments based on the Kretschmann configuration (e.g., Biacore, Uppsala, Sweden) and surface plasmon resonance imaging (e.g., GWC Technologies, Madison, WI, or Horiba, Kyoto, Japan) are readily available in the market, and have well established protocols to attach DNA to their surface, both in a single spot and in multiplexed array patterns. In the Kretschmann configuration, a metal film, typically gold is evaporated onto the side of a prism and incident radiation is launched at an angle to excite the surface plasmons. An evanescent wave penetrates through the metal film exciting plasmons on the other side, where it may be used to monitor near-surface and surface interactions near the gold film. At the resonant angle, the light reflected from the prism-gold interface is severely attenuated. Assuming fixed wavelength illumination, binding interactions may be examined by monitoring both the intensity of the reflected light at a fixed angle close to the resonant angle, as well as by monitoring the changes in angle of incidence required to establish surface plasmon resonance conditions (minimum reflectivity). When a 2D imaging device such as a CCD or CMOS camera is utilized to monitor the reflected light, the entire illumination area may be imaged with high resolution. This method is called surface plasmon resonance imaging (SPRi). It allows high throughput analysis of independent regions on the surface simultaneously. Broadband illumination may also be used, in a fixed angle configuration, wherein the wavelength that is coupled to the surface plasmon resonance is identified spectroscopically by looking for dips in the reflected spectrum. Surface interactions are monitored through shifts in the resonant wavelength.

**[0234]** Surface plasmon resonance is well established for monitoring protein-nucleic acid interactions, and there exist many standard protocols both for nucleic acid attachment as well for analyzing the data. Illustrative references from literature include Bongsup Cho et al.,

"Binding Kinetics of DNA-Protein Interaction Using Surface Plasmon Resonance," Protocol Exchange, May 22,2013, and, Jennifer M. Brockman, Anthony G. Frutos, and Robert M. Corn, "A Multistep Chemical Modification Procedure To Create DNA Arrays on Gold Surfaces for the Study of Protein-DNA Interactions with Surface Plasmon Resonance Imaging," Journal of the American Chemical Society 121, no. 35 (September 1, 1999): 8044-51, both of which are included herein in their entireties by reference.

**[0235]** Polymerase/nucleic-acid interactions may be monitored on nanostructured surfaces capable of sustaining localized surface plasmons. Optionally, polymerase/nucleic-acid interactions may be monitored on nanostructured surfaces capable of sustaining surface plasmon polaritons.

**[0236]** Optionally, polymerase/nucleic-acid interactions may be monitored on nanostructured surfaces capable of sustaining localized surface plasmons. Optionally, polymerase/nucleic-acid interactions may be monitored on nanostructured surfaces capable of sustaining surface plasmon polaritons.

**[0237]** Optionally, extraordinary optical transmission (EOT) through a nanoholes array may be used to monitor nucleic-acid/polymerase interactions. Light transmitted across subwavelength nanoholes in plasmonic metal films is higher than expected from classical electromagnetic theory. This enhanced optical transmission may be explained by considering plasmonic resonant coupling to the incident radiation, whereby at resonant wavelength, a larger than anticipated fraction of light is transmitted across the metallic nanoholes. The enhanced optical transmission is dependent on the dimensions and pitch of the nanoholes, properties of the metal, as well as the dielectric properties of the medium on either side of the metal film bearing the nanoholes. In the context of a biosensor, the transmissivity of the metallic nanohole array depends on the refractive index of the medium contacting the metal film, whereby, for instance, the interaction of polymerase with nucleic acid attached to the metal surface may be monitored as a change in intensity of light transmitted across the nanoholes array. The elegance of the EOT/plasmonic nanohole array approach is that the instrumentation and alignment requirements of surface plasmon resonance may be replaced by very compact optics and imaging elements. For instance, just a low power LED illumination and inexpensive CMOS or CCD camera may suffice to implement robust EOT plasmonic sensors. An exemplary nanohole array-based surface plasmon resonance sensing device is described in C. Escobedo et al., "Integrated Nanohole Array Surface Plasmon Resonance Sensing Device Using a Dual-Wavelength Source," Journal of Micromechanics

and Microengineering 21, no. 11 (November 1, 2011): 115001, which is herein incorporated by reference in its entirety.

**[0238]** The plasmonic nanohole array may be patterned on an optically opaque layer of gold (greater than 50 nm thickness) deposited on a glass surface. Optionally, the plasmonic nanohole array may be patterned on an optically thick film of aluminum or silver deposited on glass. Optionally, the nanohole array is patterned on an optically thick metal layer deposited on low refractive index plastic. Patterning plasmonic nanohole arrays on low refractive index plastics enhances the sensitivity of the device to refractive index changes by better matching the refractive indices on the two sides of the metal layer. Optionally, refractive index sensitivity of the nanohole array is increased by increasing the distance between holes. Optionally, nanohole arrays are fabricated by replication, for example, by embossing, casting, imprint-lithography, or template-stripping. Optionally, nanohole arrays are fabricated by self-assembly using colloids. Optionally, nanohole arrays are fabricated by projection direct patterning, such as laser interference lithography.

**[0239]** A nano-bucket configuration may be preferable to a nanohole configuration. In the nanohole configuration, the bottom of the nano-feature is glass or plastic or other appropriate dielectric, whereas in the nano-bucket configuration, the bottom of the nano-feature comprises a plasmonic metal. The nanobucket array configuration may be easier to fabricate in a mass production manner, while maintaining the transmission sensitivity to local refractive index.

**[0240]** Optionally, the nanohole array plasmonic sensing is combined with lens-free holographic imaging for large area imaging in an inexpensive manner. Optionally, a plasmonic biosensing platform comprises a plasmonic chip comprising nanohole arrays, a light- emitting diode source configured to illuminate the chip, and a CMOS imager chip to record diffraction patterns of the nanoholes, which is modulated by molecular binding events on the surface. The binding events may be the formation of a ternary complex between a polymerase and a template nucleic acid in the presence of a nucleotide.

**[0241]** The methods to functionalize surfaces (for nucleic acid attachment) for surface plasmon resonance sensing may be directly applied to EOT nanohole arrays as both sensing schemes employ similar metal surfaces to which nucleic acids need to be attached.

**[0242]** Optionally, the refractive index changes associated with polymerase/nucleic acid interaction may be monitored on nanostructured surfaces that do not support plasmons. Optionally, guided mode resonance may be used to monitor the polymerase/nucleic-acid interaction. Guided-mode resonance or waveguide-mode resonance is a phenomenon wherein

the guided modes of an optical waveguide can be excited and simultaneously extracted by the introduction of a phase-matching element, such as a diffraction grating or prism. Such guided modes are also called "leaky modes," as they do not remain guided and have been observed in one and two-dimensional photonic crystal slabs. Guided mode resonance may be considered a coupling of a diffracted mode to a waveguide mode of two optical structured placed adjacent or on top of each other. For instance, for a diffraction grating placed on top of an optical waveguide, one of the diffracted modes may couple exactly into the guided mode of the optical waveguide, resulting in propagation of that mode along the waveguide. For off-resonance conditions, no light is coupled into the waveguide, so the structure may appear completely transparent (if dielectric waveguides are used). At resonance, the resonant wavelength is strongly coupled into the waveguide, and may be coupled out of the structure depending on downstream elements from the grating-waveguide interface. In cases where the grating coupler is extended over the entire surface of the waveguide, the light cannot be guided, as any light coupled in is coupled out at the next grating element. Therefore, in a grating waveguide structure, resonance is observed as a strong reflection peak, whereas the structure is transparent to off-resonance conditions. The resonance conditions are dependent on angle, grating properties, polarization and wavelength of incident light. For cases where the guided mode propagation is not present, for instance due to a grating coupled to the entire surface of the waveguide, the resonant mode may also be called leaky-mode resonance, in light of the strong optical confinement and evanescent propagation of radiation in a transverse direction from the waveguide layer. Change in dielectric properties near the grating, for instance due to binding of biomolecules affects the coupling into the waveguide, thereby altering the resonant conditions. Optionally, where nucleic acid molecules are attached to the surface of grating waveguide structures, the polymerase/nucleic-acid interaction may be monitored as a change in wavelength of the leaky mode resonance.

**[0243]** A diffraction element may be used directly on a transparent substrate without an explicit need for a waveguide element. The change in resonance conditions due to interactions near the grating nanostructure may be monitored as resonant wavelength shifts in the reflected or transmitted radiation.

**[0244]** Reflected light from a nucleic acid attached guided mode resonant sensor may be used to monitor the polymerase/nucleic-acid interaction. A broadband illumination source may be employed for illumination, and a spectroscopic examination of reflected light could reveal changes in local refractive index due to polymerase binding.

**[0245]** Optionally, a broadband illumination may be used and the transmitted light may be examined to identify resonant shifts due to polymerase interaction. A linearly polarized narrow band illumination may be used, and the transmitted light may be filtered through a cross-polarizer; wherein the transmitted light is completely attenuated due to the crossed polarizers excepting for the leaky mode response whose polarization is modified. This implementation converts refractive index monitoring to a simple transmission assay that may be monitored on inexpensive imaging systems. Published material describe the assembly of the optical components. See, Yousef Nazirizadeh et al., "Low-Cost Label-Free Biosensors Using Photonic Crystals Embedded between Crossed Polarizers," Optics Express 18, no. 18 (August 30, 2010): 19120-28, which is incorporated herein by reference in its entirety.

**[0246]** In addition to nanostructured surfaces, plain, unstructured surfaces may also be used advantageously for monitoring refractive index modulations. Optionally, interferometry may be employed to monitor the interaction of polymerase with nucleic acid bound to an unstructured, optically transparent substrate. Nucleic acid molecules may be attached to the top surface of a glass slide (by any means known in the art), and the system illuminated from the bottom surface of the glass slide. There are two reflection surfaces in this configuration, one reflection from the bottom surface of the glass slide, and the other from the top surface which has nucleic acid molecules attached to it. The two reflected waves may interfere with each other causing constructive or destructive interference based on the path length differences, with the wave reflected from the top surface modulated by the changes in dielectric constant due to the bound nucleic acid molecules (and subsequently by the interaction of polymerase with the bound nucleic acid molecules). With the reflection from the bottom surface unchanged, any binding to the nucleic acid molecules may be reflected in the phase difference between the beams reflected from the top and bottom surfaces, which in turn affects the interference pattern that is observed. Optionally, bio-layer interferometry is used to monitor the nucleic acid/polymerase interaction. Bio-layer interferometry may be performed on commercial devices such as those sold by Pall Forte Bio corporation (Menlo Park, CA).

**[0247]** Optionally, the reflected light from the top surface is selectively chosen by using focusing optics. The reflected light from the bottom surface is disregarded because it is not in the focal plane. Focusing only on the nucleic-acid-attached top surface, the light collected by the focusing lens comprises a planar wave, corresponding to the partially reflected incident radiation, and a scattered wave, corresponding to the radiations scattered in the collection direction by molecules in the focal plane. These two components may be made to interfere if

the incident radiation is coherent. This scattering based interferometric detection is extremely sensitive and can be used to detect down to single protein molecules.

**[0248]** Optionally, a field-effect transistor (FET) is configured as a biosensor for the detection of a ternary complex. A gate terminal of the FET is modified by the addition of template nucleic acids. The binding of a polymerase comprising a charged tag results in changes in electrochemical signals. Binding of a polymerase with a next correct nucleotide to the template nucleic acid provides different signals than polymerase binding to a template nucleic acid in the presence of other incorrect nucleotides, where each incorrect nucleotide may also provide a different signal. Optionally, polymerase interactions with a template nucleic acid are monitored using FET without the use of a labeled tag on the polymerase, primed template nucleic acid, or nucleotide. Optionally, the pH change that occurs due to release of H+ ions during the incorporation reaction is detected using a FET. Optionally, the polymerase comprises a tag that generates continuous H+ ions that is detected by the FET. Optionally, the continuous H+ ion generating tag is an ATP synthase. Optionally, the continuous H+ ion generation tag is palladium, copper or another catalyst. Optionally, the release of a PPi after nucleotide incorporation is detected using FET. For example, one type of nucleotide may be provided to a reaction at a time. Once the next correct nucleotide is added and conditions allow for incorporation, PPi is cleaved, released, and detected using FET, therefore identifying the next correct nucleotide and the next base. Optionally, template nucleic acids are bound to walls of a nanotube. Optionally, a polymerase is bound to a wall of a nanotube. FET is advantageous for use as a sequencing sensor due to its small size and low weight, making it appropriate for use as a portable sequencing monitoring component. Details of FET detection of molecular interactions are described in Dong-Sun Kim et al., "An FET-Type Charge Sensor for Highly Sensitive Detection of DNA Sequence," Biosensors and Bioelectronics, Microsensors and Microsystems 2003, 20, no. 1 (July 30, 2004): 69-74, doi:10.1016/j.bios.2004.01.025 and Alexander Star et al., "Electronic Detection of Specific Protein Binding Using Nanotube FET Devices," Nano Letters 3, no. 4 (April 1, 2003): 459-63, doi:10.1021/nl0340172, which are incorporated by reference herein in their entireties.

**[0249]** By way of example, the polymerase comprises a fluorescent tag. To monitor polymerase-nucleic acid interaction with high signal-to-noise, evanescent illumination or confocal imaging may be employed. The formation of a ternary complex on localized template nucleic acids may be observed as an increased fluorescence compared to the background, for instance, whereas in some instances it may be also be observed as a decreased fluorescence due to quenching or change in local polar environment. Optionally, a

fraction of polymerase molecules may be tagged with a fluorophore while another fraction may be tagged with a quencher in the same reaction mixture; wherein, the formation of ternary complex on a localized, clonal population of nucleic acid is revealed as decrease in fluorescence compared to the background. The clonal population of nucleic acids may be attached to a support surface such as a planar substrate, microparticle, or nanoparticle. Optionally, a polymerase is tagged with a fluorophore, luminophore, chemiluminophore, chromophore, or bioluminophore.

**[0250]** Optionally, a plurality of template nucleic acids is tethered to a surface and one (or more) dNTPs are flowed in sequentially. The spectrum of affinities reveals the identity of the next correct nucleotide and therefore the next base in the template nucleic acid. Optionally, the affinities are measured without needing to remove and replace reaction mixture conditions, i.e., a wash step. Autocorrelation of the measured intensities of the binding interaction, for instance, could readily reveal the dynamics of the interaction, thus revealing the affinities without requiring a washing step to measure the off-rate.

**[0251]** Any technique that can measure dynamic interactions between a polymerase and nucleic acid may be used to measure the affinities and enable the sequencing reaction methods disclosed herein.

Systems for Detecting Nucleotide-Specific Ternary Complex Formation

**[0252]** The provided methods can be performed on a platform, where any component of the nucleic acid polymerization reaction is localized to a surface. Optionally, the template nucleic acid is attached to a planar substrate, a nanohole array, a microparticle, or a nanoparticle. Optionally, all reaction components are freely suspended in the reaction mixture.

**[0253]** Optionally, the template nucleic acid is immobilized to a surface. The surface may be a planar substrate, a hydrogel, a nanohole array, a microparticle, or a nanoparticle. Optionally, the first reaction mixture comprises a plurality of clonally amplified template nucleic acid molecules. Optionally, the first reaction mixture comprises a plurality of distinguishable template nucleic acids.

**[0254]** Provided herein, inter alia, are systems for performing sequencing reactions involving the examination of the interaction between a polymerase and a primed template nucleic acid in the presence of nucleotides to identify the next base in the template nucleic acid sequence or to identify the number of nucleotides needed to fill a homopolymer region encountered during sequencing. Optionally, examination includes monitoring the affinity of the

polymerase for the primed template nucleic acid in the presence of nucleotides. Optionally, the polymerase binds transiently with the nucleic acid and the binding kinetics and affinity provides information about the identity of the next base on the template nucleic acid. Optionally, a closed-complex is formed, wherein the reaction conditions involved in the formation of the closed-complex provide information about the next base on the nucleic acid. Optionally, the polymerase is trapped at the polymerization site in its ternary complex by one or a combination of means, not limited to, crosslinking of the polymerase domains, crosslinking of the polymerase to the nucleic acid, allosteric inhibition by small molecules, uncompetitive inhibitors, competitive inhibitors, non-competitive inhibitors, and denaturation; wherein the formation of the trapped polymerase complex provides information about the identity of the next base on the nucleic acid template.

**[0255]** Also provided is a system for performing one or more steps of any sequencing method disclosed herein. Optionally, the system includes components and reagents necessary to perform a polymerase and template nucleic acid binding assay in the presence of nucleotides, wherein the template nucleic acid is provided on a nanostructure. Optionally, the system comprises one or more reagents and instructions necessary to bind template DNA molecules onto a nanostructure. For example, the system provides a nanostructure, such as a chip, configured for use with surface plasmon resonance to determine binding kinetics. An example of such a chip is a CM5 sensor S chip (GE Healthcare, Piscatawany, N.J. The system may provide instrumentation such as an SPR instrument (e.g., Biacore). The system may provide streptavidin and/or biotin. Optionally, the system provides biotin-DNA, DNA ligase, buffers, and/or DNA polymerase for preparation of biotinylated template DNA. Optionally, the system provides a gel or reagents (e.g., phenol:chloroform) for biotinylated DNA purification. Alternatively, the system provides reagents for biotinylated template DNA characterization, for example, mass spectrometry or HPLC. Optionally, the system comprises streptavidin, a chip, reagents, instrumentation, and/or instructions for immobilization of streptavidin on a chip. Optionally, a chip is provided in the system already configured for template DNA coating, wherein the chip is immobilized with a reagent capable of binding template nucleic acids or modified template nucleic acids (e.g., biotinylated template DNA). Optionally, the system provides reagents for chip regeneration.

**[0256]** Also provided is a system for performing one or more steps of any sequencing method disclosed herein. Optionally, the system includes components and reagents necessary to perform a polymerase and template nucleic acid binding assay in the presence of nucleotides, wherein the template nucleic acid is provided on a nanoparticle. Optionally, the system

comprises one or more reagents and instructions necessary to bind template DNA molecules onto a nanoparticle. The nanoparticle may be configured for the electrochemical detection of nucleic acid-polymerase interaction, for instance, by using gold nanoparticles. Optionally, the DNA-nanoparticle conjugates are formed between aqueous gold colloid solutions and template DNA molecules comprising free thiol or disulfide groups at their ends. The conjugates may comprise clonally amplified populations of DNA molecules which may or may not comprise the same nucleic acid sequence. Optionally, the nanoparticle conjugates are stabilized against flocculation and precipitation at high temperature (e.g.,> 60°C) and high ionic strength (e.g., 1M Na+). Optionally, the system provides reagents for preparing template DNA molecules for nanoparticle attachment, including, generating template DNA molecules with disulfides or thiols. Disulfide-containing template nucleic acids may be synthesized using, for example, a 3'-thiol modifier controlled-pore glass (CPG) or by beginning with a universal support CPG and adding a disulfide modifier phosphoramidite as the first monomer in the sequence. The system may provide for nucleic acid synthesis reagents and/or instructions for obtaining disulfide-modified template nucleic acids. Thiol-containing template nucleic acids may also be generated during nucleic acid synthesis with a 5'-tritylthiol modifier phosphoramidite. The system may provide reagents and/or instructions for nanoparticle conjugate purification using for example, electrophoresis or centrifugation. Optionally, nanoparticle conjugates are used to monitor polymerase-template nucleic acid interactions colorimetrically. In this instance, the melting temperature of the nanoparticle conjugate increases in the presence of strong polymerase binding. Therefore, the strength of DNA binding can be determined by the change in this melting transition, which is observable by a color change.

**[0257]** Also provided is a system for performing one or more steps of any sequencing method disclosed herein. Optionally, the system includes components and reagents necessary to perform a polymerase and template nucleic acid binding assay in the presence of nucleotides, using a detectable polymerase. Optionally, the polymerase is detectably labeled. Optionally, the polymerase is detected using intrinsic properties of the polymerase, for example, aromatic amino acids. Optionally, the polymerase and template nucleic acids are configured for use in solution, without conjugation to a support. The detectable label on the polymerase may be a fluorophore, wherein fluorescence is used to monitor polymerase- template nucleic acid binding events. Optionally, the detectable polymerase may be used in combination with template nucleic acids in solution, or template nucleic acids conjugated to a support structure. Optionally, one or more cysteine residues of the polymerase is labeled with Cy3-maleimide

or Cy3-iodoacetamide. Optionally, the system comprises reagents and/or instructions necessary to prepare fluorescently labeled polymerase molecules. The system may comprise reagents and/or instructions for purification of fluorescently labeled polymerases.

Procedural Features of the Methods

**[0258]** Following the examination step, where the identity of the next base has been identified via formation of a ternary complex, the reaction conditions may be reset, recharged, or modified as appropriate, in preparation for the optional incorporation step or an additional examination step. Optionally, the identity of the next base has been identified without chemically incorporating a nucleotide. Optionally, the identity of the next base is identified with chemical incorporation of a nucleotide, wherein a subsequent nucleotide incorporation has been inhibited. Optionally, all components of the examination step, excluding the template nucleic acid being sequenced, are removed or washed away, returning the system to the pre-examination condition. Optionally, partial components of the examination step are removed. Optionally, additional components are added to the examination step.

**[0259]** Optionally, reversible terminator nucleotides are used in the incorporation step to ensure one, and only one nucleotide (i.e., only a single nucleotide) is incorporated per cycle. No labels are required on the reversible terminator nucleotides as the base identity is known from the examination step. Non-fluorescently labeled reversible terminators are readily available from commercial suppliers. Non-labeled reversible terminator nucleotides are expected to have much faster incorporation kinetics compared to labeled reversible terminators due to their smaller steric footprint, and similar size to natural nucleotides.

**[0260]** Disclosed herein, in part, are reagent cycling sequencing methods, wherein sequencing reagents are introduced, one after another, for every cycle of examination and/or incorporation. Optionally, the sequencing reaction mixture comprises a polymerase, a primed template nucleic acid, and at least one type of nucleotide. Optionally, the nucleotide and/or polymerase are introduced cyclically to the sequencing reaction mixture. Optionally, the sequencing reaction mixture comprises a plurality of polymerases, primed template nucleic acids, and nucleotides. Optionally, a plurality of nucleotides and/or a plurality of polymerases are introduced cyclically to the sequencing reaction mixture. Optionally, the examination step of the sequencing reaction has a different composition than the incorporation step of the sequencing reaction.

**[0261]** As provided herein, a polymerase refers to a single polymerase or a plurality of polymerases. As provided herein, a primed template nucleic acid or template nucleic acid refers to a single primed template nucleic acid or single template nucleic acid, or a plurality of primed template nucleic acids or a plurality of template nucleic acids. As provided herein, a nucleotide refers to one nucleotide or a plurality of nucleotides. As provided herein, a single nucleotide is one nucleotide. Optionally, the sequencing reaction nucleotides include, but are not limited to, 1, 2, 3, or 4 of the following nucleotides: dATP, dGTP, dCTP, and dTTP (or dUTP). Optionally, reagent cycling involves immobilizing a template nucleic acid to a platform, washing away the current reaction mixture, and adding a new reaction mixture to the template nucleic acid.

**[0262]** Optionally, one or more nucleotides are sequentially added to and removed from the sequencing reaction. Optionally, 1, 2, 3, 4, or more types of nucleotides are added to and removed from the reaction mixture. For example, one type of nucleotide is added to the sequencing reaction, removed, and replaced by another type of nucleotide. Optionally, a nucleotide type present during the examination step is different from a nucleotide type present during the incorporation step. Optionally, a nucleotide type present during one examination step is different from a nucleotide type present during a sequential examination step (i.e., the sequential examination step is performed prior to an incorporation step). Optionally, 1, 2, 3, 4 or more types of nucleotides are present in the examination reaction mixture and 1, 2, 3, 4, or more types of nucleotides are present in the incorporation reaction mixture.

**[0263]** Optionally, a polymerase is cyclically added to and removed from the sequencing reaction. One or more different types of polymerases may be cyclically added to and removed from the sequencing reaction. Optionally, a polymerase type present during the examination step is different from a polymerase type present during the incorporation step. A polymerase type present during one examination step may be different from a polymerase type present during a sequential examination step (i.e. the sequential examination step is performed prior to an incorporation step).

**[0264]** Optionally, reagents conditions such as the presence of reagents, pH, temperature, and ionic strength are varied throughout the sequencing reaction. Optionally, a metal is cyclically added to and removed from the sequencing reaction. For example, a catalytic metal ion may be absent during an examination step and present during an incorporation step. Alternatively, a polymerase inhibitor may be present during an examination step and absent during an incorporation step. Optionally, reaction components that are consumed during the

sequencing reaction are supplemented with the addition of new components at any point during the sequencing reaction.

**[0265]** Nucleotides can be added one type at a time, with the polymerase, to a reaction condition which favors ternary complex formation, and destabilizes formation of binary complexes. The polymerase binds only to the template nucleic acid if the next correct nucleotide is present. A wash step after every nucleotide addition ensures all excess polymerases and nucleotides not involved in a ternary complex are removed from the reaction mixture. If the nucleotides are added one at a time, in a known order, the next base on the template nucleic acid is determined by the formation of a ternary complex when the added nucleotide is the next correct nucleotide. The ternary complex may be identified by both the conformational change and the increased stability of the polymerase-template nucleic acid-nucleotide interaction. Optionally, the stability of the ternary complex formed in the presence of the next correct nucleotide is at least an order of magnitude greater than the unstable interactions of the polymerase with the template nucleic acid in the presence of incorrect nucleotides. The use of a wash step ensures that there are no unbound nucleotides and polymerases and that the only nucleotides present in the reaction are those sequestered in a ternary complex with a polymerase and a template nucleic acid. Once the next base on the template nucleic acid is determined, the next correct nucleotide sequestered in the closed-complex may be incorporated by a reagent exchange (e.g., flowing through a flow cell) that provides reaction conditions favoring dissociation or destabilization of the ternary complex, and extending the template nucleic acid primer strand by one base (incorporation). Therefore, the wash step ensures that the only nucleotide incorporated is the next correct nucleotide from the ternary complex. This reagent cycling method may be repeated and the nucleic acid sequence determined. This reagent cycling method may be applied to a single template nucleic acid molecule, or to collections of clonal populations such as PCR products or rolling-circle amplified DNA. Many different templates can be sequenced in parallel if they are arrayed, for instance, on a solid support. Optionally, the wash step destabilizes binary complex formation. Optionally, the washing is performed for a duration of time which ensures that the binary complex is removed, leaving the stabilized closed-complex in the reaction mixture. Optionally, the wash step includes washing the reaction with a high ionic strength or a high pH solution.

**[0266]** Optionally, the incorporation step is a three stage process. In the first stage, all four nucleotide types are introduced into a reaction comprising a primed template nucleic acid, with a high fidelity polymerase, in reaction conditions which favor the formation of a ternary

complex, and the next correct nucleotides are allowed to form stable ternary complexes with the template nucleic acid. In a second stage, excess nucleotides and unbound polymerase optionally are washed away. In a third stage, reaction conditions are modified so that the ternary complex is destabilized and the sequestered nucleotides within the ternary complex become incorporated into the 3'-end of the primer of the primed template nucleic acid. Formation of tight polymerase-nucleic acid complexes in the incorporation step can be enabled by standard techniques such as fusing a non-specific DNA binding domain to the polymerase (e.g., Phusion polymerase), and utilizing high concentrations of nucleotides to ensure correct nucleotides are always present in the closed-complex.

**[0267]** Polymerase molecules bind to primed nucleic acid molecules in a fingers-closed conformation in the presence of the next correct nucleotide even in the absence of divalent metal ions that are typically required for polymerase synthesis reactions. The conformational change traps the nucleotide complementary to the next template base within the active site of the polymerase. Optionally, the formation of the ternary complex may be used to determine the identity of the next base on the template nucleic acid. Optionally, the primed template nucleic acids may be contacted serially by different nucleotides in the presence of polymerase, in the absence of catalytic divalent metal ions; wherein the formation of a closed-complex indicates the nucleotide currently in contact with the template nucleic acid is the complementary nucleotide to the next base on the nucleic acid. A known order of nucleotides (in the presence of polymerase, absence of catalytic metal ions) brought into contact with the template nucleic acid ensures facile identification of the complementary nucleotide based on the particular position in the order that induces ternary complex formation. Optionally, an appropriate wash step may be performed after every nucleotide addition to ensure removal of all excess enzymes and nucleotides, leaving behind only the polymerase that are bound to nucleic acids in a ternary complex with the next correct nucleotide at the active site. The ternary complex may be identified by means that reveal the conformational change of the polymerase in the closed conformation or by means that reveal the increased stability of the polymerase/nucleic-acid/next-correct-nucleotide complex compared to binary polymerase-nucleic acid complexes or compared to unstable interactions between the polymerase, primed template nucleic acid and incorrect nucleotides.

**[0268]** Optionally, the process of identifying the next complementary nucleotide (examination step) comprises the steps of contacting immobilized primed template nucleic acids with an examination mixture comprising polymerase and nucleotides of one kind under conditions that inhibit the chemical incorporation of the nucleotide, optionally removing

unbound reagents by a wash step, detecting the presence of polymerase ternary complex on the immobilized nucleic acids, and repeating these steps, serially, with nucleotides of different kinds until a ternary complex is formed and detected. The ternary complex may be identified by both the conformational change and the increased stability of the polymerase/nucleic-acid/next-correct- nucleotide complex. The wash step between successive nucleotide additions may be eliminated by the use of detection mechanisms that can detect the formation of the closed-complex with high fidelity, for instance, evanescent wave sensing methods or methods that selectively monitor signals from the ternary complex. The examination steps noted above may be followed by an incorporation step comprising, contacting the ternary complex with catalytic metal ions to covalently add the nucleotide sequestered in the ternary complex to the 3'-end of the primer. Optionally, the incorporation step may comprise, contacting the immobilized nucleic acids with a pre-incorporation mixture comprising a combination of multiple types of nucleotides and polymerase under conditions that inhibit the chemical incorporation of the nucleotides; wherein the pre-incorporation mixture may contain additives and solution conditions to ensure highly efficient ternary complex formation (e.g., low-salt conditions). The methods may also include performing a wash step to remove unbound reagents and providing the immobilized complexes with an incorporation mixture, comprising catalytic metal ions, to chemically incorporate nucleotides sequestered within the active site of the polymerase. The pre-incorporation mixture ensures highly efficient ternary complex formation, while the wash step and incorporation mixture ensure the addition of a single nucleotide to the 3'-end of the primer. Optionally, the incorporation step may occur directly after examination an addition of one type of nucleotide. For instance, a repeated pattern used for sequencing may comprise the following flow pattern (i) dATP+/polymerase, (ii) Wash, (iii) Mg2+, (iv) Wash, (v) dTTP+/polymerase, (vi) Wash, (vii) Mg2+, (viii) Wash, (ix) dCTP+/polymerase, (x) Wash (xi) Mg2+, (xii) Wash, (xiii) dGTP+/polymerase, (xiv) Wash, (xv) Mg2+, (xvi)Wash. Optionally, the repeated pattern used for sequencing may include (i) dATP+/polymerase, (ii) Wash, (iii) dTTP+/polymerase, (iv) Wash, (v) dGTP+/polymerase, (vi) Wash, (vii) dCTP+/polymerase, (viii) Wash, (ix) Pre-incorporation mixture, (x) Wash, (xi) Mg2+, (xii)Wash. The wash steps typically contain metal ion chelators and other small molecules to prevent accidental incorporations during the examination steps. After the incorporation step, the primer strand is typically extended by one base. Repeating this process, sequential nucleobases of a nucleic acid may be identified, effectively determining the nucleic acid sequence. Optionally, the examination step is performed at high salt conditions, for example,

under conditions of 50 mM to 1,500 mM salt (i.e., a salt providing monovalent cations, such as monovalent metal cations).

**[0269]** For sequencing applications, it can be advantageous to minimize or eliminate fluidics and reagents exchange. Removing pumps, valves and reagent containers can allow for smaller and less complicated devices. Disclosed herein, in part, are "all-in" sequencing methods, wherein the need to introduce reagents one after another, for every cycle of examination and/or incorporation, is eliminated. Reagents are added only once to the reaction, and sequencing-by-synthesis is performed by manipulating reagents already enclosed within the sequencing reaction. A scheme such as this requires a method to distinguish different nucleotides, a method to synchronize incorporation of nucleotides across a clonal population of nucleic acids and/or across different nucleic acid molecules, and a method to ensure only one nucleotide is added per cycle. Optionally, the sequencing reaction mixture includes a polymerase, a primed template nucleic acid, and at least one type of nucleotide. Optionally, the sequencing reaction mixture comprises a plurality of polymerases, primed template nucleic acids, and nucleotides. As provided herein, a polymerase refers to a single polymerase or a plurality of polymerases. As provided herein, a primed template nucleic acid or template nucleic acid refers to a single primed template nucleic acid or single template nucleic acid, or a plurality of primed template nucleic acids or a plurality of template nucleic acids. As provided herein, a nucleotide refers to one nucleotide or a plurality of nucleotides. As provided herein, a single nucleotide is one nucleotide. Optionally, the sequencing reaction nucleotides include, but are not limited to, 1, 2, 3, or 4 of the following nucleotides: dATP, dGTP, dCTP, and dTTP (or dUTP).

**[0270]** Optionally, the examination step and the incorporation step take place in a single sequencing reaction mixture.

**[0271]** Optionally, 1, 2, 3, 4 or more types of nucleotides (e.g. dATP, dGTP, dCTP, dTTP) are present in the reaction mixture together at the same time, wherein one type of nucleotide is a next correct nucleotide. The reaction mixture further comprises at least one polymerase and at least one primed template nucleic acids. Optionally, the template nucleic acid is a clonal population of template nucleic acids. Optionally, the polymerase, primed template nucleic acid, and the nucleotide form a ternary complex under examination reaction conditions.

**[0272]** In the provided methods, four types of nucleotides can be present at distinct and different concentrations wherein the diffusion and binding time of the polymerase to the template nucleic acid is different for each of the four nucleotides, should they be the next

correct nucleotide, due to the different concentrations of the four nucleotides. For example, the nucleotide at the highest concentration would bind to its complementary base on the template nucleic acid with a faster time, and the nucleotide at the lowest concentration would bind to its complementary base on the template nucleic acid with a slower time; wherein binding to the complementary base on the template nucleic acid refers to the polymerase binding to the template nucleic acid with the next correct nucleotide in a ternary complex. The identity of the next correct nucleotide is therefore determined by monitoring the rate or time of binding of polymerase to the template nucleic acid in a ternary complex. Optionally, the four types of nucleotides may be distinguished by their concentration, wherein the different concentrations of the nucleotides result in measurably different on-rates for the polymerase binding to the nucleic acid. Optionally, the four types of nucleotides may be distinguished by their concentrations, wherein the different concentrations of the nucleotides result in measurably different on-rates for the formation of a stabilized ternary complex.

**[0273]** Optionally, the polymerase is labeled. In some instances, the polymerase is not labeled and any label free detection method disclosed herein or known in the art is employed. Optionally, the binding of the polymerase to the nucleic acid is monitored via a detectable feature of the polymerase. Optionally, the formation of a stabilized ternary complex is monitored via a detectable feature of the polymerase. A detectable feature of the polymerase may include, but is not limited to, optical, electrical, thermal, colorimetric, mass, and any combination thereof.

**[0274]** Optionally, 1, 2, 3, 4, or more nucleotides types (e.g., dATP, dTTP, dCTP, dGTP) are tethered to 1, 2, 3, 4, or more different polymerases; wherein each nucleotide type is tethered to a different polymerase and each polymerase has a different tag or a feature from the other polymerases to enable its identification. All tethered nucleotide types can be added together to a sequencing reaction mixture forming a ternary complex comprising a tethered nucleotide-polymerase; the ternary complex is monitored to identify the polymerase, thereby identifying the next correct nucleotide to which the polymerase is tethered. The tethering may occur at the gamma phosphate of the nucleotide through a multi-phosphate group and a linker molecule. Such gamma-phosphate linking methods are standard in the art, where a fluorophore is attached to the gamma phosphate linker. The tags include, but are not limited to, optical, electrical, thermal, colorimetric, mass, or any other detectable feature. Optionally, different nucleotide types are identified by distinguishable tags. Optionally, the distinguishable tags are attached to the gamma phosphate position of each nucleotide.

**[0275]** Optionally, the sequencing reaction mixture comprises a catalytic metal ion. Optionally, the catalytic metal ion is available to react with a polymerase at any point in the sequencing reaction in a transient manner. To ensure robust sequencing, the catalytic metal ion is available for a brief period of time, allowing for a single nucleotide complementary to the next base in the template nucleic acid to be incorporated into the 3'-end of the primer during an incorporation step. In this instance, no other nucleotides, for example, the nucleotides complementary to the bases downstream of the next base in the template nucleic acid, are incorporated. Optionally, the catalytic metal ion magnesium is present as a photocaged complex (e.g., DM-Nitrophen) in the sequencing reaction mixture such that localized UV illumination releases the magnesium, making it available to the polymerase for nucleotide incorporation. Furthermore, the sequencing reaction mixture may contain EDTA, wherein the magnesium is released from the polymerase active site after catalytic nucleotide incorporation and captured by the EDTA in the sequencing reaction mixture, thereby rendering magnesium incapable of catalyzing a subsequent nucleotide incorporation.

**[0276]** Thus, in the provided methods, a catalytic metal ion can be present in a sequencing reaction in a chelated or caged form from which it can be released by a trigger. For example, the catalytic metal ion catalyzes the incorporation of the ternary complex next correct nucleotide, and, as the catalytic metal ion is released from the active site, it is sequestered by a second chelating or caging agent, disabling the metal ion from catalyzing a subsequent incorporation. The localized release of the catalytic metal ion from its cheating or caged complex is ensured by using a localized uncaging or un-chelating scheme, such as an evanescent wave illumination or a structured illumination. Controlled release of the catalytic metal ions may occur for example, by thermal means. Controlled release of the catalytic metal ions from their photocaged complex may be released locally near the template nucleic acid by confined optical fields, for instance by evanescent illumination such as waveguides or total internal reflection microscopy. Controlled release of the catalytic metal ions may occur for example, by altering the pH of the solution near the vicinity of the template nucleic acid. Chelating agents such as EDTA and EGTA are pH dependent. At a pH below 5, divalent cations $Mg^{2+}$ and $Mn^{2+}$ are not effectively chelated by EDTA. A method to controllably manipulate the pH near the template nucleic acid allows the controlled release of a catalytic metal ion from a chelating agent. Optionally, the local pH change is induced by applying a voltage to the surface to which the nucleic acid is attached. The pH method offers an advantage in that that metal goes back to its chelated form when the pH is reverted back to the chelating range.

**[0277]** Optionally, a catalytic metal ion is strongly bound to the active site of the polymerase, making it necessary to remove the polymerase from the template nucleic acid after a single nucleotide incorporation. The removal of polymerase may be accomplished by the use of a highly distributive polymerase, which falls off the 3'-end of the strand being synthesized (primer) after the addition of every nucleotide. The unbound polymerase may further be subjected to an electric or magnetic field to remove it from the vicinity of the nucleic acid molecules. Any metal ions bound to the polymerase may be sequestered by chelating agents present in the sequencing reaction mixture, or by molecules which compete with the metal ions for binding to the active site of the polymerase without disturbing the formation of the closed- complex. The forces which remove or move the polymerase away from the template nucleic acid (e.g., electric field, magnetic field, and/or chelating agent) may be terminated, allowing for the polymerase to approach the template nucleic acid for another round of sequencing (i.e., examination and incorporation). The next round of sequencing, in a non-limiting example, comprises the formation of a ternary complex, removing unbound polymerase away from the vicinity of the template nucleic acid and/or ternary complex, controlling the release of a catalytic metal ion to incorporate a single nucleotide sequestered within the ternary complex, removing the polymerase which dissociates from the template nucleic acid after single incorporation away from the vicinity of the template nucleic acid, sequestering any free catalytic metal ions through the use of chelating agents or competitive binders, and allowing the polymerase to approach the template nucleic acid to perform the next cycle of sequencing.

**[0278]** Described above are polymerase-nucleic acid binding reactions for the identification of a nucleic acid sequence. Nucleic acid sequence identification may include information regarding nucleic acid modifications. Nucleic acid modifications include methylation and hydroxymethylation. Methylation may occur on cytosine bases of a template nucleic acid. DNA methylation may stably alter the expression of genes. DNA methylation is also indicated in the development of various types of cancers, atherosclerosis, and aging. DNA methylation therefore can serve as an epigenetic biomarker for human disease.

**[0279]** Optionally, one or more cytosine methylations on a template nucleic acid are identified during the sequencing-by-binding methods provided herein. The template nucleic acid may be clonally amplified prior to sequencing, wherein the amplicons comprise the same methylation as their template nucleic acid. Amplification of the template nucleic acids may include the use of DNA methyltransferases to achieve amplicon methylation. The template nucleic acids or amplified template nucleic acids are provided to a reaction mixture

comprising a polymerase and one or more nucleotide types, wherein the interaction between the polymerase and nucleic acids is monitored. Optionally, the interaction between the polymerase and template nucleic acid in the presence of a methylated cytosine is different than the interaction in the presence of an unmodified cytosine. Therefore, based on examination of a polymerase-nucleic acid interaction, the identity of a modified nucleotide is determined.

**[0280]** Provided herein are methods to create a table of polymerase-nucleic acid affinities for a variety of possible combinations of nucleotides and next bases. Because these affinities are affected primarily by interactions at the polymerase active site, where only the nucleotide and next base on the nucleic acid template participate, context specific effects may be neglected. Context specific effects may include secondary structures of the nucleic acid and contribution of bases further down the sequence from the next base on the template nucleic acid. The table of affinities allows for the determination of a nucleotide, natural or modified, which induces the widest and most easily measured dispersion in affinities for different template bases. Optionally, the template bases are 1, 2, 3, or 4 of the bases dATP, dTTP, dCTP, and dGTP. It is understood that the strongest affinity will exist for the base that is complementary to the nucleotide. As used herein, dispersion particularly refers to the variation in affinities for the other three incorrect, non-complementary bases. Optionally, each affinity is measured under examination conditions that inhibit nucleotide incorporation and destabilize binary complex formation. Optionally, the polymerase is modified or selected to provide a wide dispersion in affinities. The engineered or natural polymerase may have unfavorable error profiles or other unfavorable properties as a sequencing enzyme, which is immaterial as this polymerase will only be used under non-incorporating conditions. A polymerase may be expressly selected for its ability to provide easily measurable and distinct affinities for different template bases. Evolving or screening for polymerases with desired properties are standard procedures in the art (e.g., screening for polymerases with high affinity for modified nucleotides). Optionally, a polymerase is replaced with a high-fidelity polymerase for an incorporation step. Optionally, a combination of polymerase and nucleotide is selected that provides the most convenient affinities for the template bases, thereby allowing for the execution of a sequencing method that measure the affinity of the selected polymerase to the template nucleic acid in the presence of the selected nucleotide. The next base on the nucleic acid is determined from the measured affinity, as the spectrum of affinities for the template bases is provided in the constructed affinity table. The affinity

can be an on-rate, off-rate, or combination of on-rate and off-rate of the polymerase nucleic acid interaction.

**[0281]** The affinity of a polymerase for a template nucleic acid in the presence of a nucleotide can be measured in a plurality of methods known to one of skill in the art. Optionally, the affinity is measured as an off-rate, where the off-rate is measured by monitoring the release of the polymerase from the template nucleic acid as the reaction is washed by a wash buffer. Optionally, the affinity is measured as an off-rate, where the off-rate is measured by monitoring the release of the polymerase from the template nucleic acid under equilibrium binding conditions, especially equilibrium binding conditions where the polymerase binding rates are low or diffusion limited. The polymerase binding rates may be diffusion limited at sufficiently low concentrations of polymerase, wherein if the polymerase falls-off from the DNA-polymerase complex, it does not load back immediately, allowing for sufficient time to detect that the polymerase has been released from the complex. For a higher affinity interaction, the polymerase is released from the nucleic acid slowly, whereas a low affinity interaction results in the polymerase being released more rapidly. The spectrum of affinities, in this case, translates to different off-rates, with the off-rates measured under dynamic wash conditions or at equilibrium. The smallest off-rate corresponds to the base complementary to the added nucleotide, while the other off-rates vary, in a known fashion, depending on the combination of polymerase and nucleotide selected.

**[0282]** Optionally, the off-rate is measured as an equilibrium signal intensity after the polymerase and nucleotide are provided in the reaction mixture, wherein the interaction with the lowest off-rate (highest affinity) nucleotide produces the strongest signal, while the interactions with other, varying, off-rate nucleotides produce signals of measurably different intensities. As a non-limiting example, a fluorescently labeled polymerase, measured, preferably, under total internal reflection (TIRF) conditions, produces different measured fluorescence intensities depending on the number of polymerase molecules bound to surface-immobilized nucleic acid molecules in a suitably chosen window of time. The intrinsic fluorescence of the polymerase, for instance, tryptophan fluorescence, may also be utilized. A high off-rate interaction produces low measured intensities, as the number of bound polymerase molecules, in the chosen time window is very small, wherein a high off-rate indicates that most of the polymerase is unbound from the nucleic acid. Any surface localized measurement scheme may be employed including, but not limited to, labeled or fluorescence schemes. Suitable measurement schemes that measure affinities under equilibrium conditions include, but are not limited to, bound mass, refractive index, surface

charge, dielectric constant, and schemes known in the art. Optionally, a combination of on-rate and off-rate engineering yields higher fidelity detection in the proposed schemes. As a non-limiting example, a uniformly low on-rate, base dependent, varying off-rate results in an unbound polymerase remaining unbound for prolonged periods, allowing enhanced discrimination of the variation in off-rate and measured intensity. The on-rate may be manipulated by lowering the concentration of the added polymerase, nucleotide, or both polymerase and nucleotide.

**[0283]** Optionally, the interaction between the polymerase and the nucleic acid is monitored via a detectable tag attached to the polymerase. The tag may be monitored by detection methods including, but limited to, optical, electrical, thermal, mass, size, charge, vibration, and pressure. The label may be magnetic, fluorescent or charged. For external and internal label schemes, fluorescence anisotropy may be used to determine the stable binding of a polymerase to a nucleic acid in a ternary complex.

**[0284]** By way of example, a polymerase is tagged with a fluorophore, wherein closed-complex formation is monitored as a stable fluorescent signal. The unstable interaction of the polymerase with the template nucleic acid in the presence of an incorrect nucleotide results in a measurably weaker signal compared to the ternary complex formed in the presence of the next correct nucleotide.

**[0285]** Optionally, the interaction between the polymerase and the template nucleic acid in the presence of a nucleotide is monitored utilizing intrinsic signals from the polymerase including, but not limited to, Raman signature, tryptophan, 2-aminopurine or other intrinsic fluorescence. Intrinsic fluorescence of polymerase amino acids can be exploited to detect polymerase conformational changes that occur during one or more steps of a sequencing method disclosed herein, for example ternary complex formation or release. Amino acids with intrinsic fluorescence include tryptophan, tyrosine, and phenylalanine. Optionally, one or more polymerase amino acids are mutated to comprise a tryptophan, tyrosine, or phenylalanine residue. Polymerases may be modified by any means, including genetic or chemical modification, to comprise additional amino acids with intrinsic fluorescence. Optionally, intrinsic fluorescence is influenced by the proximity of other amino acids which may cause quenching of fluorescence, such as those amino acids having protonated groups (e.g., aspartic acid, glutamic acid). Optionally, a tryptophan residue of a polymerase is buried in a hydrophobic core, when the polymerase is configured in a ternary complex, and exposed to an aqueous environment, when the polymerase is released or not engaged in a closed-complex confirmation. The emission spectrum of the tryptophan is different

depending on the environment (hydrophilic or hydrophobic), allowing for the detection of a ternary complex. Optionally, intrinsic fluorescence of a polymerase is used to identify conformational changes during a sequencing reaction. In one example, intrinsic fluorescence emissions from tryptophan residues of a polymerase are monitored using techniques similar to those referenced in Yu-Chih Tsai, Zhinan Jin, and Kenneth A. Johnson, "Site-Specific Labeling of T7 DNA Polymerase with a Conformationally Sensitive Fluorophore and Its Use in Detecting Single- Nucleotide Polymorphisms," Analytical Biochemistry 384, no. 1 (January 1, 2009): 136-44, which is herein incorporated in its entirety by reference.

**[0286]** Optionally, in the provided methods, following one or more examination and/or incorporation steps a subset of nucleotides is added to reduce or reset phasing. Thus, the methods can include one or more steps of contacting a template nucleic acid molecule being sequenced with a composition comprising a subset of nucleotides and an enzyme for incorporating the nucleotides into the strand opposite the template strand of the nucleic acid molecule. The contacting can occur under conditions to reduce phasing in the nucleic acid molecule. Optionally, the step of contacting the template nucleic acid molecule occurs after an incorporation step and/or after an examination step. Optionally, the contacting occurs after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, or 100 rounds or more of sequencing, i.e., rounds of examination and incorporation. Optionally, the contacting occurs after 30 to 60 rounds of sequencing. Optionally, the contacting occurs after every round of sequencing, i.e., after one examination and incorporation step. Optionally, multiple contacting steps occur after every round of sequencing, wherein each contacting step may comprise different subsets of nucleotides. Optionally, the method further comprises one or more washing steps after contacting. Optionally, the subset comprises two or three nucleotides. Optionally, the subset comprises three nucleotides. Optionally, the subset of nucleotides is selected from three of dATP, dGTP, dCTP, dTTP (or dUTP) or a derivative thereof. Optionally, the three nucleotides comprise adenosine, cytosine, and guanine. Optionally, the three nucleotides comprise adenosine, cytosine, and thymine. Optionally, the three nucleotides comprise cytosine, guanine and thymine. Optionally, the three nucleotides comprise adenosine, guanine and thymine. Optionally, each round of contacting comprises the same subset or different subsets of nucleotides. Optionally, sequencing of a nucleic acid template is monitored and the contacting with the subset of nucleotides occurs upon detection of phasing. See also for example, U.S. Patent No. 8,236,532, which is incorporated herein by reference in its entirety.

**[0287]** Optionally, the sequencing reaction involves a plurality of template nucleic acids, polymerases and/or nucleotides, wherein a plurality of ternary complexes is monitored. Clonally amplified template nucleic acids may be sequenced together wherein the clones are localized in close proximity to allow for enhanced monitoring during sequencing. Optionally, the formation of a ternary complex ensures the synchronicity of base extension across a plurality of clonally amplified template nucleic acids. The synchronicity of base extension allows for the addition of only one base per sequencing cycle.

Examples

**[0288]** Example 1 describes procedures that investigated the consequence of including or omitting a divalent catalytic cation during the examination step. The procedure was conducted using a label-free system and Klenow polymerase.

**Example 1**

Determination of Base Sequence With or Without Magnesium in the Binding Step

**[0289]** Materials and methods used in the procedure were as follows. Polymerase buffer: 20 mM Tris, pH 8.0, 300 mM NaCl, 5mM DTT, 100 μM dNTP, 150 nM Klenow, 0.01% BSA, 0.02% Tween-20, 10 mM $MgCl_2$. Exam buffer: 20 mM Tris buffer (pH 8.0), 300 mM NaCl, 5 mM DTT, 100 μM dNTP, 150 nM Klenow, 0.01% BSA, 0.02% Tween-20. Incorporation buffer: 20 mM Tris buffer (pH 8), 300 mM NaCl, 5 mM DTT, 0.01% BSA, 0.02% Tween-20, 10 mM $MgCl_2$. Wash Buffer: 20 mM Tris buffer (pH 8), 300 mM NaCl, 5 mM DTT, 0.01% BSA, 0.02% Tween-20.

**[0290]** Figure 1 shows the results of an experiment using non-labeled optical detection methods where magnesium was present or absent during the examination step. The first flow included dCTP (C:T mismatch) and the second flow included dATP (A:T match). The solid line in Figure 1 shows the results obtained using Polymerase Buffer. The pre-steady state rate constants were 0.0106 and 0.0084 for the match A and mismatch C steps, respectively. The difference was too small to accurately discriminate the cognate base. The dashed line in Figure 1 represents a magnesium-free examination step in exam buffer, followed by soaking in incorporation buffer. A signal threshold of 1.1 nm accurately determined the correct base. These results showed that the sensing platform was unable to capture transient kinetics that could discriminate a match from mismatch base, and would be incapable of sequence determination when magnesium was included in the buffer during examination (Polymerase

Buffer, solid line, Figure 1). In contrast, binding in the absence of magnesium provided very large discrimination between correct and incorrect base (Exam Buffer, dashed line, Figure 1). The correct base sequence in this procedure was determined by signal thresholding rather than binding rates.

**[0291]** Example 2 describes procedures that demonstrated how binding kinetics can be used to determine the correct base or nucleotide during the examination step (*i.e.*, during the formation of a ternary complex between the polymerase, DNA template and nucleotide). The Bst enzyme showed a bimodal binding curve when the correct base was presented and a basic exponential binding behavior when the incorrect base was presented, thereby allowing for discrimination and detection of the correct base or nucleotide during the procedure.

## Example 2

Sequencing Using Bst Enzyme Binding Kinetics

**[0292]** Materials and methods used in the procedure were as follows. The FORTEBIO® (Menlo Park, CA) Octet instrument (Red384 or QK) uses biolayer interferometry to measure binding reactions at the surface of a fiber optic tip. The tips were functionalized with streptavidin (SA) to enable binding to 5' biotin labeled DNA templates hybridized with a primer that is complementary to sequences near the 3'-end of the template. PhiX_matchC and phiX_matchA were loaded onto individual tips. Primer-template was loaded onto the tips at between 100 and 500 nM in 1-2x PBS containing 0.01-0.02% BSA and 0.01-0.02% Tween-20 (loading buffer). The FP2 primer was in 1.25 - 2 fold excess over template. Loading was monitored by a change in signal, and usually reached a plateau within 5 minutes at 30°C. Tips were soaked in loading buffer for 1-5 minutes to remove unbound DNA material. For base calling, tips were typically soaked in solutions containing 1X Taq buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, at 25°C, magnesium-free) supplemented with 0.01-0.02% BSA and 0.01-0.02% Tween-20 (LS buffer), 100 nM polymerase enzyme, 100 μM nucleotide, and varying concentrations of additional NaCl from 50 to 300 mM. In this experiment, for determining the correct base, 30 nM Bst2.0 enzyme, 100 μM dNTP, and LS buffer containing 150 mM NaCl was used. The phiX_matchC duplex will form a ternary complex and show an increase in binding signal because the correct next nucleotide (cognate) is presented. The phiX_matchA should not because it is an incorrect nucleotide (noncognate). After the examination step, the sensors were soaked in LS buffer containing 5

mM $Mg^{2+}$, to allow the polymerase to incorporate the nucleotide, which was followed by washing with LS buffer containing 150 mM NaCl.

**[0293]** Results of the procedure are shown in Figure 2. Iterative cycling showed that this method can be used for sequence determination. Table 2 shows that the first three bases were correctly identified by this examination method. The fourth base was a "no call" by the examination method, and may reflect multiple additions. Consistent with this, subsequent bases were correctly identified. Overall, 5 of 6 bases were identified correctly. Further, misincorporation of an incorrect base was not observed.

**Table 2**
Base Identification

| Expected base | A | G | C | T | Comments |
|---|---|---|---|---|---|
| C | x | x | C | | |
| G | x | G | | | |
| C | x | x | C | | |
| C | x | x | ? | | Difficult call |
| T | x | x | x | T | |
| T | X | x | x | T | |

**[0294]** Example 3 describes a sequencing reaction wherein an examination step that employed high salt conditions was followed by an incorporation step.

## Example 3

Sequencing-by-Binding

**[0295]** Materials and methods used in the procedure were as follows. The binding/examination buffer used in this instance was LS buffer having 250 mM NaCl, 100 µM dGTP, dCTP, dATP, or dTTP, 1.5 mM $Sr^{2+}$, 100 nM Klenow exo(-) polymerase. The incorporation buffer was LS buffer with 50 mM NaCl, 50 mM $MgCl_2$, and the wash buffer was LS buffer with 250 mM NaCl. Using a FORTEBIO® Octet Red384 system (Menlo Park, CA), sequencing cycles were performed using biotin phiX_matchC template and FP2 primer sequences attached to SA sensor tips. Sequencing steps consisted of the following: (a) Examination with dATP, (b) incorporation, (c) wash; (d) Examination with dGTP, (e) incorporation, (f) wash; (g) Examination with dCTP, (h) incorporation, (i) wash; (j) Examination with dTTP (k) incorporation, (l) wash, followed by repeat of these steps from

(a). For base calls, the examination step produced a detectable signal above the baseline from the previous wash step and/or relative to a control sequences.

**[0296]** Results from this procedure indicated that 12 bases were correctly identified. Two bases were not identified because the binding signal was too low. This experiment identified 12/14 bases correctly, as shown below in Table 3.

**Table 3**

Sequence Identification

| Expected base | G | C | T | A | Comment |
|---|---|---|---|---|---|
| C | | C | | | |
| G | G | | | | |
| C | | C | | | |
| C | | | | | No call |
| T | | | T | | |
| T | | | T | | |
| C | | C | | | |
| G | G | | | | |
| T | | | T | | |
| A | | | | A | |
| T | | | | | No call |
| G | G | | | | |
| T | | | T | | |
| T | | | T | | |

**[0297]** Example 4 describes procedures initially used to establish the effect of a salt containing a monovalent cation on polymerase match/mismatch discrimination. The FORTEBIO® Octet instrument (Red384 or QK) (Menlo Park, CA) employed in the procedure used biolayer interferometry to measure binding reactions at the surface of a fiber optic tip. The tips had been functionalized with streptavidin (SA) to enable binding to 5' biotin labeled DNA templates hybridized with a primer that was complementary to sequences near the 3'-end of the template.

**Example 4**

Salt Concentration on Match/Mismatch Base Discrimination

**[0298]** Materials and methods used in the procedure were as follows. PhiX_matchC and phiX_matchA were loaded onto individual tips. Primer-template was loaded onto the tips at between 100 and 500 nM in 1-2xPBS containing 0.01-0.02% BSA and 0.01-0.02% Tween-20 (loading buffer). The FP2 primer was in 1.25 - 2 fold excess over template. Loading was

monitored by a change in signal, and usually reached a plateau within 5 minutes at 30°C. Tips were soaked in Loading buffer for 1-5 minutes to remove unbound DNA material. For base calling, tips were typically soaked in solutions containing 1X Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 at 25°C, magnesium free) supplemented with 0.01-0.02% BSA and 0.01-0.02% Tween-20 (LS buffer), 100 nM polymerase enzyme, 100 μM dNTP, and varying concentrations of additional NaCl from 50 to 300 mM. The phiX_matchC duplex will form a ternary complex and show an increase in binding signal, because the correct next nucleotide (cognate) is presented. The phiX_matchA should not cause an increased binding signal because it is an incorrect (noncognate) nucleotide.

**[0299]** Results indicated that both templates bound polymerase enzyme under standard reaction conditions. However, as the salt concentration increased, the binding affinity of the non-cognate complex decreased while binding affinity of the cognate complex remained high. Thus, the signal to noise ratio (SNR) of base discrimination increased such that the next correct base can be easily identified during this examination step (see Figure 3). Although NaCl was used in this example as the model salt containing a monovalent cation, other salts such as KCl, NH2(SO4), potassium glutamate, and others known in the art also can be used. Polymerases that show differences in binding affinity between correct and incorrect nucleotides included Klenow polymerase, Bst2.0, Bsu, and Taq.

**[0300]** Example 5 describes procedures that demonstrated how examination during both the association and dissociation phases of the binding reaction could be used to improve sequence fidelity. In this instance, the cognate nucleotide could be identified by dissociation of (i.e., loss of) a ternary complex.

## Example 5

Base Discrimination During Dissociation/Wash Step

**[0301]** Materials and methods used in the procedure were as follows. In this experiment, phiX_matchC and FP2 primer were loaded onto SA-sensor tips, as described above. Polymerase complex was formed in LS buffer with 100 μM of either dGTP, dCTP, dATP, or dTTP, and 100 nM Klenow or Bst2.0 enzyme, and 1 mM $SrCl_2$.

**[0302]** Results indicated that, in low salt, polymerase efficiently bound to DNA of the primed template nucleic acid regardless of whether cognate nucleotide was present. In wash buffer (LS buffer + 50 mM or 100 mM added NaCl), all complexes dissociated. Even $SrCl_2$ did not

stabilize complexes when additional NaCl was present. However, when 50 μM of the same dNTP that was in the binding buffer was included in the wash buffer, then only the complexes with incorrect nucleotides dissociated and the correct ternary complex was stabilized (*see* Figure 4). Furthermore, it was found that dNTPs were unnecessary in the binding step and could be included during washing. A bound binary complex could still allow the correct base to enter and form a ternary complex when the correct dNTP was subsequently introduced. Additionally, fidelity was not affected by the presence of incorrect nucleotides. Thus, the dissociation rates of the polymerase can also be used to determine the correct base in a mixture of dNTPs (*e.g.*, at different concentrations which will dissociate at different rates).

## Example 6

Stabilization of Nucleic Acid:Polymerase Complex in Wash Buffer

**[0303]** To minimize the possibility of multiple incorporations in a homopolymer region, a wash step can be performed before incorporation. Metal cations such as calcium and strontium can stabilize the polymerase complex (like magnesium), but cannot support catalysis of the phosphodiester bond which results in incorporation of the nucleotide. In this experiment, varying concentrations of $SrCl_2$ (0 mM – 14 mM) were added to the wash buffer (LS buffer). The polymerase complex was much more stable in the presence of as little as 3.5 mM $Sr^{2+}$ ion (lowest concentration) in the wash buffer. Further, stability of the complex was not noticeably affected when correct or incorrect nucleotide was present during the binding step indicating that $Sr^{2+}$ related stability of the polymerase was not limited to ternary complexes. The results are shown in Figure 5.

**[0304]** Example 7 describes procedures that investigated the effect of the 3'-5' exonuclease activity of DNA Pol I was investigated. Exonuclease activity increase fidelity as an incorrect base or flap is removed by the proofreading function provided by the exonuclease activity of the polymerase. However, this activity can potentially cleave correct bases and lead to incorrect sequence reads.

## Example 7

Use of DNA pol I Without 3'-5' Exonuclease Activity

**[0305]** Materials and methods used in the procedure were as follows. A primer with a 3' terminal mismatch was hybridized to template creating a frayed end or flap construct.

Klenow exo(-) polymerase will not be able to extend this terminus. However, DNA pol I large fragment has exonuclease activity and could remove the mismatch base. Once removed, the primer can be extended normally by klenow exo(-) polymerase or DNA polymerase. Two sensors having immobilized flap structures were exposed to either DNA polymerase or Klenow exo(-) for sequencing. The sensors were then exposed to the template sequence in the correct order. The DNA polymerase sensor was able to add the bases, whereas the Klenow fragment sensor was unable to add any bases due to the flap structure. Any 3'-5' exo activity of the DNA polymerase would cause base addition to be out of sync with the sequence.

**[0306]** Results from the procedure are presented in Figure 6. Cleavage of mismatch base by DNA polymerase allowed subsequent base additions. Bases were correct out to 4 cycles. Without exonuclease activity, Klenow exo(-) was unable to extend the template. In cases where spurious exonuclease activity is detrimental, the exonuclease can be inhibited by competitive, uncompetitive or noncompetitive compounds or analogs. For example, NaF is competitive inhibitor of DNA polymerase exonuclease function (Potapova et al., FEBS Letters, 277(1-2):109-111 (1990)).

**Table 4**

Nucleic Acid Sequences

| **Nucleic acid sequences used in Examples 2-7** | | | |
|---|---|---|---|
| Name | Length | Sequence (5'-3') | Modification |
| phiX_matchC | 101 | GGC AAA TCA CCA GAA GGC GGT TCC TGA ATG AAT GGG AAG CCT TCA AGA AGG TGA TAA GCA GGA GAA ACA TAC GAA GGC GCA TAA CGA TAC CAC TGA CCC TC (SEQ ID NO:3) | 5' biotin |
| phiX_matchA | 101 | GGC AAA TCA CCA GAA GGC GGT TCC TGA ATG AAT GGG AAG CCT TCA AGA AGG TGA TAA GCA GGA GAA ACA TAC GAA GCA TCA TAA CGA TAC CAC TGA CCC TC (SEQ ID NO:4) | 5' biotin |
| FP2 | 22 | GAG GGT CAG TGG TAT CGT TAT G (SEQ ID NO:5) | |
| PhiX_50matchA | 50 | TGA TAA GCA GGA GAA ACA TAC GAA GCA TCA TAA CGA TAC CAC TGA CCC TC (SEQ ID NO:6) | 5' biotin |
| Alk_Btn-4460-4509S | 50 | GTGAGCCTGCAATCCCTGCCCCGGTTCATC CTGCTGGAGCTCATGGCGGG (SEQ ID NO:7) | 5' biotin |
| ALK_4496-4509AS | 14 | CCCGCCATGAGCTC (SEQ ID NO:8) | |

**[0307]** Example 8 describes procedures employing HIV-1 reverse transcriptase in combination with enzyme inhibitors. As background on the target sequence used in this Example, the EML4-ALK fusion is found in 4-5% of patients with non–small-cell lung cancer (Soda et al., *Nature* 448:561-6 (2007); Mano, *Cancer Sci.* 99:2349-55 (2008); and Horn and Pao, *J. Clin. Oncol.* 27:4232-5 (2009)). The ALK C4493A mutation has been identified in clinical lung tumors, which results in the L1196M "gatekeeper" mutation in ALK protein and confers resistance to the chemotherapy drug crizotinib (Choi et al., *N. Engl. J. Med.* 18:1734-9 (2010)). The 4496-4509AS primer enables sequencing into the region with the gatekeeper mutation. Template oligonucleotide sequence was derived from wild-type human ALK gene (nucleotide numbers 4460-4509). The primer sequence was complementary to a portion of the human ALK gene (nucleotide numbers 4496-4509).

## Example 8

Sequencing Using HIV Reverse Transcriptase (RT) and Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) Compounds 7 and 18

**[0308]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:9). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). Template oligonucleotide Btn-4460-4509S and primer oligonucleotide 4496-4509AS were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. Primer and template oligonucleotides were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). The tube containing primer-template solution was loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal the strands by gradually cooling to ambient temperature. Uncompetitive NNRTI compounds (Pitta et al., J. Enzyme Inhib. Med. Chem. 28(10):113-22 (2013)), which is incorporated by reference herein in its entirety), were from Epigen Biosciences, Inc. (San Diego, CA). NNRTI compounds 7 (3-4-chloro-benzo[d]thiazol-2yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one) and 18 (3-(6-ethoxy-benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one) were dissolved in

dimethylsulfoxide (DMSO) to concentrations of 25.0 mM and 15.0 mM, respectively. Recombinant purified HIV reverse transcriptase (HIV RT) was from Worthington Biochemical Corp (Lakewood, NJ). Ultra-pure bovine serum albumin (BSA) was from Ambion (Foster City, CA). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 μM) into Annealing Buffer. Immediately before use, HIV Reverse Transcriptase was pre-diluted into Enzyme Diluent (50 mM Tris buffer (pH 8.0), 8 mM MgCl2). Binding buffer (50 mM Tris buffer (pH 8.0), 160 mM KCl, 0.5 mM EDTA, 11 mM MgCl2, 0.3% (v/v) Triton X-100, 5.3 mM dithiothreitol (DTT), 100 μg/mL bovine serum albumin (BSA), 100 μM dNTP (dATP, dTTP, dGTP or dCTP), 100 nM HIV RT). NNRTI compounds were pre-diluted with DMSO immediately before being spiked into Binding Buffer. Reaction Buffer was Binding Buffer without NNRTI, dNTP or HIV RT enzyme. Buffer containing Primer-Template (PT), PT Wash Buffer, Binding buffer containing one dNTP, and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685). Streptavidin (SA) Biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5019) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with PT Wash Buffer. Biosensors were transferred to Binding Buffer containing HIV RT and dCTP ± NNRTI (association phase) followed by Reaction Buffer (dCTP incorporation and dissociation phase). Similarly, biosensors were transferred to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) in a cycical fashion, as indicated. Cycles of binding and incorporation were repeated multiple times. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display. The progress of binding and dissociation reactions was smoothed either by averaging within a 19.4-second window or by Prism software (19.4-second window and 6th-order smoothing polynomial).

**[0309]** Results from the procedure are shown in Figures 7A-7B, and in Figure 8. Non-nucleoside reverse transcriptase inhibitors (NNRTI) with reportedly an uncompetitive mode of inhibition were utilized for DNA sequencing via the DNA-dependent DNA polymerase activity of HIV reverse transcriptase. In assays for binding to biosensor coated with primer-template, the combination of HIV reverse transcriptase, correct dNTP and NNRTI compound 7 (40 μM) exhibit distinct peaks for binding in the association phase followed by decreased

binding in wash buffer during the dissociation phase (Figure 7A, circles). Unlike the NNRTI-stabilized HIV RT-dNTP mixtures, reactions containing HIV RT and correct dNTP did not produce appreciable binding peaks (Figure 7A, triangles) nor did controls (HIV RT with or without 40 μM NNRTI compound 7; Figure 7A, solid and dashed lines). The time course for binding and dissociation demonstrate sequencing for the first six cycles (nucleotide sequence CAGCAG) in Figure 7A. The seventh cycle and eighth cycles with incorrect nucleotides dCTP and dATP, respectively did not produce a binding peak. The ninth cycle with the correct nucleotide dGTP (Figure 7A). Time courses for binding (0-5 minutes) and dissociation (5-10 minutes) are shown for each cycle in Figure 7B. Similarly, sequencing using HIV RT and NNRTI compound 18 also produced sequencing results. In assays for binding to biosensor coated with primer-template, the combination of HIV reverse transcriptase, correct dNTP and NNRTI compound 18 (80 μM) exhibit distinct peaks for binding in the association phase followed by decreased binding in wash buffer during the dissociation phase (Figure 8, diamonds). Unlike the NNRTI-stabilized HIV RT-dNTP mixtures, reactions containing HIV RT and correct dNTP did not produce appreciable binding peaks (Figure 8, triangles) nor did controls (HIV RT with 80 μM NNRTI compound 18; Figure 8, dashed lines). In cycles 1-3, binding peaks indicated binding of HIV RT with correct nucleotide and compound 18 for sequence CAG (Figure 8). Cycle 4 with HIV RT, incorrect nucleotide dTTP and compound 18 did not show a binding peak (Figure 8). Subsequent cycles did not show further peaks for sequencing analysis.

**[0310]** Example 9 describes procedures that demonstrated utility of another label-free sequencing system. More specifically, the results demonstrated the ability to accurately sequence DNA using a Klenow/dNTP binding assay on an SPRi biosensor. The SPRi biosensor has sufficient sensitivity and durability to detect the different steps necessary for performing DNA sequencing over multiple examination/incorporation rounds. Below there is shown sequencing of three base pairs within a 60 bp strand. This technique can be extendable to an arbitrary number of sequencing cycles.

## Example 9

DNA Sequencing on a Surface Plasmon Resonance (SPR) Imaging Biosensor

**[0311]** Materials and methods used in the procedure were as follows. SPR sensor chip: 20 mm x 20 mm x 1 mm high refractive index (1.61) slide (NanoSPR, Chicago, IL). Alkanethiols, PEG6: monothioalkane(C11)PEG6-OH(11-mercaptoundecyl

hexaethyleneglycol (catalogue number, SPT-0011P6); and BAT: biotinylated alkane PEG thiol (catalogue number, SPT-0012D), were obtained from Sensopath technologies (Bozeman, MT). Base buffer (wash): 300 mM KCl, 20 mM Tris HCl (pH 8.0), 0.01% Tween-20, 1 mM SrCl2. Examination buffer: Base buffer plus 50 nM Klenow fragment and 100 nM dNTP. Incorporation buffer: Base buffer plus 10 mM MgCl2. Prior to the experiment the gold coated SPR slide was coated with a mixed SAM of 18% BAT with 82% PEG6 diluted in 100% EtOH to final combined concentration of 1 x 10-4 M. SPR slides were incubated in the alkanethiol solution overnight at room temperature. After incubation the SPR slides were rinsed in fresh 100% EtOH, followed by 50% EtOH in deionized water, and deionized water. The slides were then dried in air. The slides were mounted on a custom built SPR imaging system that provided fluidic control, image acquisition, and data quantitation. A solution containing 10 µg/ml of streptavidin in base buffer was injected into the flow cell. Binding of the resulting streptavidin layer was monitored by measuring the change in light reflected from the SPR chip for approximately 180 seconds. This was followed by washing with excess base buffer. Prehybridized primer FP2/PhiX_matchA template DNA was then injected into the flow cell and allowed to bind to the streptavidin layer for approximately 180 seconds, followed by washing with excess base buffer. For sequencing, solutions containing 50 nM Klenow fragment were prepared in base buffer with 100 nM of either dATP, dCTP, dTTP, or dGTP. The dNTP solutions were individually injected into the flow cell (in the order G, A, A, T, C, G), and allowed to incubate for approximately 180 seconds in order to examine the SPRi response. If there was no/low signal change, then the flow cell was washed with excess base buffer. If the SPR signal indicated a base match, then the flow cell was washed with incorporation buffer (containing 10 mM Mg2+) for 30 seconds to incorporate the correct dNTP, followed by wash with base buffer. The examination, incorporation, and wash steps were repeated.

**[0312]** Figure 9 shows the sensorgram recorded for the identification of three mismatched bases and three correct bases. The first three correct conjugate bases of the phiX_matchA template after the annealed primer sequence were A, T, and G. The first solution flowed over the sensor contained polymerase and dGTP, which corresponded to a base pair mismatch. The resulting sensor trace showed little change in baseline reflectance, indicating that the polymerase molecule did not bind to the primed template strand. The next solution flowed over the sensor contained polymerase and dATP, which corresponded to the correct conjugate base. The resulting trace (highlighted in the gray box), showed a strong increase in reflected light indicating that the polymerase had bound to the primed template strand,

thereby shifting the position of the SPR. After allowing the polymerase solution to incubate for approximately 180 seconds (to ensure saturation of available binding sites), incorporation solution containing 10 mM Mg2+ was introduced into the flow cell. The introduction of Mg2+ allowed the polymerase to incorporate the bound dATP into the primer strand bound to the template. To ensure successful incorporation of dATP, the polymerase-dATP solution was again flowed over the sensor chip. This time, however, the amount of the reflected light did not increase as strongly as before indicating that the polymerase did not bind to the template strand as the correct cognate base was no longer A. To examine the next correct base, a solution of polymerase and dTTP was flowed over the sensor. Once again the intensity of reflected light increased above the threshold value indicating that the incorporation of dATP was successful and the next correct base was T, as expected. Incorporation buffer was flowed over the sensor chip to incorporate the base. The process was repeated two more time using a mismatch (polymerase-dCTP), followed by a match (polymerase-dGTP). In both cases the expected response was observed indicating that the next correct conjugate base was in fact G.

**[0313]** Example 10 describes procedures that demonstrated sequencing of a double-stranded DNA template.

## Example 10

Sequencing Double-Stranded DNA by Nick Translation

**[0314]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S having a 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:9). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). The DNA sequence of oligonucleotide 4460-4494AS was: 5'-AGCAGGATGAACCGGG/i5NitInd/CAGGGATTGCAGGCTCAC-3' (SEQ ID NO:11), where "/i5NitInd/" is a 5-nitroindole-2'-deoxyribose residue. 5-Nitroindole is intended to prevent formation of guanine tetrads in this context and serves as a universal base. Oligonucleotides were prepared in TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides Btn-4460-4509S and 4496-4509AS were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). To prepare the dsDNA primer/template with a 1-base pair gap, oligonucleotides Btn-4460-4509S, 4496-4509AS and

4460-4494AS were combined (10 μM each strand) in a tube containing Annealing Buffer. The tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Full-length DNA polymerase encoded by Bacillus stearothermophilus ("Bst DNA polymerase", recombinant enzyme purified from Escherichia coli) was purchased from New England Biolabs (Ipswich, MA; catalog no. M0328L). Ultra-pure bovine serum albumin (BSA) was purchased from Ambion (Foster City, CA). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 μM) into Annealing Buffer. Binding buffer was 50 mM Tris buffer (pH 8.0), 300 mM KCl, 0.1% (v/v) Triton-X100, 100 μg/mL bovine serum albumin. Reaction Buffer was Binding Buffer containing 10 mM MgCl2. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP, and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). Streptavidin (SA) Biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5019) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Binding Buffer. Biosensors were transferred to Binding Buffer containing 136 Unit/mL Bst DNA Polymerase and 200 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by Reaction Buffer for dNTP incorporation. Biosensors were transferred to Reaction Buffer containing Bst DNA Polymerase (136 Unit/mL) without dNTP to promote nick translation via 5'-3' exonuclease activity. Biosensors were transferred to Reaction Buffer without enzyme or magnesium (dissociation phase). Similarly, biosensors were transferred to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) in a cyclical fashion, as indicated. Cycles of binding and incorporation and 5'-3' exonucleolytic cleavage were repeated multiple times to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0315]** Results from the procedure are shown in Figures 10A-10C. In assays for binding to biosensor coated with primer-template, the combination of Bst DNA polymerase and correct 2'-deoxyribonucleoside triphosphate (dCTP) exhibit distinct peak for binding in the association phase (Figs. 10A, 10B, and 10C, dCTP "C"). Cycle 1 demonstrates binding and

incorporation to the one base pair gap in the dsDNA template (Figs. 10A and 10B) and incorporation of correct nucleotide downstream of the primer in the control ssDNA template (Figure 10C). Controls lacking dNTP show minimal binding (Figs. 10A, 10B, and 10C, cycles 1-7). Cycle 2 shows binding of polymerase to dsDNA template in combination with the next correct nucleotide, dATP (Figs. 10A and 10B, dATP "A"), which is less than that of the unobstructed ssDNA template (Figure 10C, dATP "A") indicates that the exonucleolytic cleavage of the complementary strand was not complete. The time course for binding and dissociation demonstrate sequencing for the first three cycles (nucleotide sequence CAG) of dsDNA (Figure 10A and 10B) and ssDNA (Figure 10C). As expected the fourth cycle with the incorrect nucleotide dTTP did not produce a binding peak (Figs. 10A, 10B, and 10C). These results demonstrate the ability to sequence double-stranded DNA by nick translation using the DNA polymerase and 5'-3' exonuclease activities of Bst DNA polymerase.

**[0316]** Example 11 describes additional procedures used to demonstrate sequencing on a double-stranded DNA template.

## Example 11

Sequencing Double-Stranded DNA by Strand Displacement

**[0317]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:9). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). The DNA sequence of oligonucleotide 4460-4494AS was: 5'-AGCAGGATGAACCGGG/i5NitInd/CAGGGATTGCAGGCTCAC-3' (SEQ ID NO:11), where "/i5NitInd/" is a 5-nitroindole-2'-deoxyribose residue. 5-Nitroindole is intended to prevent formation of guanine tetrads in this context and serves as a universal base. The DNA sequence of oligonucleotide 4460-4494AS-T8 was: 5'-TTTTTTTTAGCAGGATGAACCGGG/i5NitInd/CAGGGATTGCAGGCTCAC-3' (SEQ ID NO:12), where "/i5NitInd/" is a 5-nitroindole-2'-deoxyribose residue. 5-Nitroindole is intended to prevent formation of guanine tetrads in this context and serves as a universal base. Oligonucleotides Btn-4460-4509S, 4460-4494AS, 4496-4509AS and 4460-4494AS-T8 were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA)

to 100 μM. To prepare the ssDNA primer/template, oligonucleotides Btn-4460-4509S and 4496-4509AS were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). To prepare the dsDNA primer/template with a 1-base gap, oligonucleotides Btn-4460-4509S, 4496-4509AS and 4460-4494AS were combined (10 μM each strand) in a tube containing Annealing Buffer. To prepare the dsDNA primer/template with a 5'-oligo-dT flap and 1-base gap, oligonucleotides Btn-4460-4509S, 4496-4509AS and 4460-4494-AS-T8 were combined (10 μM each strand) in a tube containing Annealing Buffer. The tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradual cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Ultra-pure bovine serum albumin (BSA) was purchased from Ambion (Foster City, CA). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (50 nM) into Annealing Buffer. Binding buffer was 20 mM Tris, pH 8.0, 300 mM KCl, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Reaction Buffer was Binding Buffer containing 50 mM KCl and 10 mM MgCl2. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP, and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). Streptavidin (SA) Biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5019) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Binding Buffer. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by Reaction Buffer for dNTP incorporation. Biosensors were transferred to Reaction Buffer without enzyme or magnesium (dissociation phase). Similarly, biosensors were transferred cyclically to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) as indicated. Cycles of binding and incorporation were repeated multiple times to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0318]** Results from the procedure are shown in Figures 11A-11C. In assays for binding to biosensor coated with ssDNA primer-template, Klenow exo(-) yielded binding peaks in the presence of the correct individual dNTPs but not with the incorrect dTTP nucleotide (Figure 11A, dark trace). By contrast, the negative control (enzyme without dNTP) failed to bind as shown by a consistently flat binding response (Figure 11A, gray trace). Thus, Klenow exo(-) yielded a sequence of CAGCAG (C?)A (SEQ ID NO:13), which is 88% identical to the expected sequence (CAGCAGGA (SEQ ID NO:1)). In assays for binding to biosensor coated with dsDNA primer-template with a 1-base gap between the anti-sense primer and the downstream antisense strand, Klenow exo(-) yielded a binding peak only in the presence of the first correct individual dCTP nucleotide (Figure 11B, dark trace). The negative control (enzyme without dNTP) failed to bind as shown by a consistently flat binding response (Figure 11B, gray trace). Thus, Klenow exo(-) yielded a sequence of "C" in which the first base of the gap is filled with the correct nucleotide. However, in subsequent cycles further binding or polymerization into the double-stranded DNA region is blocked. Blockage of further sequencing is likely due to the inherent lack of the 5'-3' exonuclease domain of Klenow exo(-) (for nick translation) or to inability to disrupt the downstream double-stranded helix of DNA (strand displacement). Therefore, a 5'-oligo-dT flap was introduced to provide a better substrate to Klenow exo(-) for strand displacement. Biosensors were coated with dsDNA primer-template bearing a 5'-oligo-dT flap with a 1-base gap between the anti-sense primer and the downstream antisense strand. Klenow exo(-) yielded binding peaks in the 1-base gap (C) of cycle #1, and additional binding peaks were observed into the dsDNA region providing the sequence AGC for cycle #2, 3 and 5 (Figure 11C, dark trace). Cycles #4, 6, 7 and 8 with the incorrect dNTP did not afford binding of enzyme to the immobilized DNA (Figure 11C). The negative control (enzyme without dNTP) failed to bind as shown by a flat binding response (Figure 11C, gray trace). Thus, Klenow exo(-) yielded a 100% correct sequence of "CAGC" in which the first base of the gap was filled with the correct nucleotide, and further binding or polymerization was observed for three additional bases into the double-stranded DNA region. The 5'-flap adjacent to the double-stranded DNA region enabled Klenow exo(-) to sequence by strand displacement (Figure 11C), whereas lack of the 5'-flap blocked sequencing into the dsDNA region (Figure 11B). These results demonstrate the ability to sequence double-stranded DNA using Klenow exo(-) fragment of DNA polymerase by a strand displacement mechanism.

**[0319]** Example 12 describes procedures that investigated the effect of glutamate anions on the sequencing reaction.

## Example 12

Effect of Anions on Single Stranded DNA Sequencing

**[0320]** To prepare the ssDNA primer/template, oligonucleotides Btn-4460-4509S (SEQ ID NO:9) and 4496-4509AS (SEQ ID NO:10) were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA, 80 mM KCl). Tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Potassium glutamate was purchased from (Teknova, Hollister, CA; catalog no. P2000). Ultra-pure bovine serum albumin (BSA) was purchased from Ambion (Foster City, CA). All reagents and solutions were molecular biology grade. Binding buffer was 20 mM Tris buffer (pH 8.0), 300 mM KCl, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Reaction Buffer was Binding Buffer containing 50 mM KCl and 10 mM MgCl2. For each level of potassium glutamate tested, the Binding Buffers were prepared to contain 0, 50, 100 or 200 mM potassium glutamate. Reaction Buffers did not contain potassium glutamate. The Octet QK biosensor system was set up as described in Example 11 Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by Reaction Buffer for dNTP incorporation. Biosensors were transferred to Reaction Buffer containing magnesium without enzyme (dissociation phase). Biosensors were transferred to Binding Buffers without enzyme or dNTP but containing the respective concentrations of potassium glutamate to re-equilibrate. Similarly, biosensors were transferred to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) in a cycical fashion, as indicated. Cycles of binding and incorporation were repeated multiple times to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0321]** Results of the procedure are shown in Figures 12A-12C. In assays for binding to biosensor coated with ssDNA primer-template, Klenow exo(-) (KCl without glutamate) yielded binding peaks for sequencing using the correct nucleotides as did enzyme in KCl containing 200 mM glutamate (Figure 12A, dotted line and solid line, respectively). However, buffers without glutamate exhibited decreased amplitude of binding peaks

combined with increasing background compared to formulations with 200 mM glutamate (Figure 12A). Correct sequence was observed in KCl and 200 mM glutamate formulations according to the relative peak heights for mixtures containing enzyme and correct dNTP (but not incorrect dNTP) over a 8.25-hour time course (Figure 12A). Homopolymer runs appear to be detected as a single peak under these conditions. (Figures 12A, 12B, and 12C). In buffers containing KCl and 100 mM glutamate, correct sequences were observed with strong peak signal for enzyme and correct dNTP over the course of 7 hours, whereas the control (enzyme without dNTP) produced no peaks and a gradual increase in background (Figure 12B). Buffers containing KCl and 50 mM glutamate show correct sequences over the course of 7 hours, whereas the control enzyme without dNTP yielded no binding peaks and a flat, stable background (Figure 12C). These results demonstrate the ability to sequence single-stranded DNA using Klenow exo(-) fragment of DNA polymerase with enhancement by potassium glutamate and protection from evaporation by mineral oil overlay.

**[0322]** Example 13 describes procedures used to demonstrate detection of small quantities of mutant sequence in the presence of similar sequences using ssDNA or dsDNA templates.

## Example 13

### Detecting a Point Mutation in a Wild-Type Background by Sequencing Single-Stranded DNA and Double-Stranded 5'-Flap DNA

**[0323]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:9). The DNA sequence of template oligonucleotide Btn-4460-4509S C4493A with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGATGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:21). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). The DNA sequence of oligonucleotide 4460-4494AS-T8 was: 5'-TTTTTTTTAGCAGGATGAACCGGG/i5NitInd/CAGGGATTGCAGGCTCAC-3' (SEQ ID NO:12), where "/i5NitInd/" is a 5-nitroindole-2'-deoxyribose residue. 5-Nitroindole is intended to prevent formation of guanine tetrads in this context and serves as a universal base. Oligonucleotides Btn-4460-4509S, Btn-4460-4509S C4493A, 4496-4509AS and 4460-4494AS-T8 were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-

MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides Btn-4460-4509S (or C4493A) and 4496-4509AS were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). To prepare the dsDNA primer/template with a 5'-oligo-dT flap and 1-base gap, oligonucleotides Btn-4460-4509S (or C4493A), 4496-4509AS and 4460-4494-AS-T8 were combined (10 μM each strand) in a tube containing Annealing Buffer. The tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 min), and the block was transferred to the bench top to anneal strands by gradual cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). Nickel(II) sulfate hexahydrate (catalog no. 467901), dCDP, dGDP and dTDP were purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (50 nM) into Annealing Buffer. Wash buffer was 20 mM Tris, pH 8.0, 200 mM KCl, 200 mM potassium glutamate, 0.01% (v/v), Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was Wash Buffer containing 2.0 mM Ni(II)SO4. Reaction Buffer was 20 mM Tris, pH 8.0, 50 mM KCl, MgCl2 (10 mM), 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. EDTA Wash Buffer was Binding Buffer containing 100 μM EDTA. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), Ni(II)SO4 (1.5 mM) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by dNTP incorporation (dissociation phase) in Reaction Buffer containing MgCl2 (10 mM). Biosensors were transferred to EDTA Wash Buffer followed by re-equilibration in Reaction Buffer without enzyme, nucleotide or divalent cation. Similarly,

biosensors were transferred to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) in a cycical fashion, as indicated. Cycles of binding and incorporation were repeated for each dNTP to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0324]** Results of the procedure are shown in Figures 13A-13C. In assays for binding to biosensor coated with ssDNA primer-template, Klenow exo(-) enzyme bound strongly to the biosensor in the presence of correct dNTP in cycles 1-2 (Figure 13A). The "G" peak in cycle 3 shows increasing peak height in mixtures containing increasing concentrations of ALK wild-type template (Figure 13A). The "T" peak in cycle 4 shows increasing peak heights in mixtures containing increasing concentrations of ALK C4493A mutation. The "T" readout at cycle 4 corresponds to the antisense nucleotide of the C4493A mutant. Both ALK wild-type and C4493A templates produce full-height peaks of "C" and "A" in cycles 5 and 6. Peaks indicate correct sequences for ALK wild-type (CAGCA) and ALK C4493A (CATCA) in Figure 13A. Peak intensities during sequencing allow quantitation of mutant allele in mixtures with wild-type sequence. The intensity of cycle 4 peak (T) was proportional to the quantity of ALK C4493A mutant sequence in the ALK wild-type background for ssDNA (Figure 13B) and dsDNA-flap (Figure 13C). Similarly, the cycle 3 peak (G) decreased linearly with increasing mutant concentration in the ssDNA template (Figure 13B), and peak 3 intensity decreased with mutant concentration for the dsDNA-flap template (Figure 13C). In a wild-type background, as little as 5% of a mutant sequence could be detected in either ssDNA or dsDNA-flap templates.

**[0325]** Example 14 describes procedures that were used to demonstrate stabilization of ternary complexes by different divalent cations.

## Example 14

Effect of Divalent Cations on Stabilizing the Ternary Complex and Polymerase Catalysis

**[0326]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S C4493A with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGATGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:21). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). Oligonucleotides Btn-4460-4509S C4493A and 4496-4509AS were synthesized and analyzed (liquid chromatography-mass spectrometry

(LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides Btn-4460-4509S C4493A and 4496-4509AS were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). The tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). Strontium chloride, calcium chloride, manganese chloride, barium chloride, cobalt chloride, zinc chloride, copper(II) chloride, ferrous ammonium sulfate, ammonium sulfate, nickel(II) sulfate hexahydrate, dCDP, dGDP and dTDP were purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 nM) into Annealing Buffer. Wash buffer was 20 mM Tris buffer (pH 8.0), 50 mM KCl, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was 20 mM Tris buffer (pH 8.0), 200 mM KCl, 200 mM potassium glutamate, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Reaction Buffer was 20 mM Tris buffer (pH 8.0), 50 mM KCl, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol and the indicated divalent cation. EDTA Wash Buffer was Binding Buffer containing 100 μM EDTA. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. To measure the initial level of unincorporated primer/template, biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), SrCl2 (2.0 mM) and 100 μM dCTP (association phase) followed by Wash Buffer containing SrCl2 (2.0 mM) and salmon sperm DNA trap (500 μg/mL) without MgCl2. Sensors were transferred to Wash Buffer containing and salmon sperm DNA trap (500 μg/mL) without

MgCl2, followed by EDTA Wash Buffer and re-equilibration in Binding Buffer. Binding to unincorporated primer/template was measured and stability of the ternary complex in divalent cation-free Binding Buffer was monitored. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), either CoCl2 (1.0 mM) or the indicated divalent cation (2.0 mM), and 100 μM dCTP (association phase) followed by Wash Buffer containing the same concentration of the same divalent cation and salmon sperm DNA trap (500 μg/mL) without MgCl2. Sensors were transferred to Wash Buffer containing and salmon sperm DNA trap (500 μg/mL) without MgCl2, followed by EDTA Wash Buffer and re-equilibration in Binding Buffer. Stabilization of the ternary complex in Binding Buffers containing various divalent cations without dNTP was measured. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), either CoCl2 (1.0 mM) or the indicated divalent cation (2.0 mM), and 100 μM dCTP (association phase) followed by Wash Buffer containing the same divalent cation and salmon sperm DNA trap (500 μg/mL) without MgCl2. Sensors were transferred to Wash Buffer containing and salmon sperm DNA trap (500 μg/mL) with 10 mM MgCl2, followed by EDTA Wash Buffer and re-equilibration in Binding Buffer. Finally, nucleotide incorporation was determined by measuring the remaining unincorporated primer/template. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), SrCl2 (2.0 mM) and 100 μM dCTP (association phase) followed by Wash Buffer containing SrCl2 (2.0 mM) and salmon sperm DNA trap (500 μg/mL) without MgCl2. Sensors were transferred to Wash Buffer containing and salmon sperm DNA trap (500 μg/mL) without MgCl2, followed by EDTA Wash Buffer and re-equilibration in Binding Buffer. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0327]** Results of the procedure are shown on Figures 14A-14B. Assays were performed for binding of polymerase and dCTP to biosensor coated with ssDNA primer-template. In the first non-catalytic cycle, Klenow exo(-) enzyme bound strongly to the biosensor in the presence of correct nucleotide (dCTP) and SrCl2 followed by wash that returns to baseline (Figure 14A, Peak#1). In the second (non-catalytic) cycle, the sensors are bound strongly by enzyme and dCTP in the presence of Ni(II)SO4, BaCl2 and SrCl2 but not EDTA (Figure 14 A, Peak#2-3). Wash Buffer containing Ni(II)SO4 stabilizes the ternary complex over the course of 10 minutes (Figure 14A, Peak#2-3). Signal decreases in the presence of Mg2+ (Figure 14A, Peak#3 dissociation) which corresponds to incorporation as evidenced by low levels of Sr2+-mediated binding by Klenow exo(-) and dCTP (Figure 14A, Peak #4). These results demonstrate the ability of Ni2+ to stabilize the ternary complex of Klenow exo(-),

dNTP and ssDNA primer/template in buffers lacking dNTP, and this stabilization is compatible with enzymatic incorporation of nucleotide by the DNA polymerase. Klenow exo(-) exhibits polymerase activity in the presence of alternative divalent cations other than magnesium ion. Assays were performed for binding of polymerase and dCTP to biosensor coated with ssDNA primer-template. In the first non-catalytic cycle, Klenow exo(-) enzyme bound strongly to the biosensor in the presence of correct nucleotide (dCTP) and SrCl2 followed by wash that returns to baseline (Figure 14B, Peak#1). In the second binding cycle, the sensors are bound strongly by enzyme and dCTP in the presence of SrCl2 or ammonium sulfate (Figure 14B, Peak#2-3). However, several divalent cations (Cu2+, Ca2+, Co2+, Fe2+, Zn2+) displayed a transient peak for binding immobilized primer/template by Klenow exo(-) and dCTP in the Binding Buffer (Figure 14B, Peak 2). Following the disappearance of the transient peak (Ca2+, Co2+, Fe2+, Zn2+) or the flat peak (Mn2+), Wash Buffer containing the same divalent cations and salmon sperm DNA trap or trap alone did not further decrease the binding signal (except for Ca2+ brought to baseline) in Figure 14A (Peak#2). In cycle 3, a second exposure of biosensors to enzyme, dCTP and the divalent cations Ca2+, Co2+, Fe2+, Zn2+ did not exhibit appreciable binding (Figure 14B, Peak 3), nor did the standard Sr2+-mediated binding control (Figure 14B, Peak 4). To a lesser extent, Cu2+ also appears to enhance polymerase activity by Klenow exo(-), because the second exposure to Cu2+ (Figure 14B, Peak 3) was less than the first Cu2+ binding (Figure 14B, Peak 2). This lack of binding signal for divalent cations (Ca2+, Co2+, Fe2+, Zn2+, Mn2+) after the first exposure to enzyme and dNTP and failure of Sr2+–mediated control conditions to support binding suggests that complete nucleotide incorporation was achieved using certain divalent cations (Ca2+, Co2+, Fe2+, Zn2+, Mn2+) in the absence of Mg2+, and these transient peaks can be used for DNA sequencing.

**[0328]** Example 15 describes procedures employing the Co2+ divalent cation.

## Example 15

Long Read-Lengths by Sequencing Single-Stranded DNA using $CoCl_2$-Mediated Binding and Catalysis

**[0329]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide phiX_100 mismatch was: Biotin-5'-
GGCAAATCACCAGAAGGCGGTTCCTGAATGAATGGGAAGCCTTCAAGAA-
GGTGATAAGCAGGAGAAACATACGAAGCATCATAACGATACCACTGACCC -3'

(SEQ ID NO:22). The DNA sequence of primer oligonucleotide FP2 was: 5'-GAGGGTCAGTGGTATCGTTATG-3' (SEQ ID NO:5). Oligonucleotides were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides "phiX_100mismatch" and "FP2" were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA, 80 mM KCl). The tube containing oligonucleotide solutions was loaded onto a dry heat block (95°C for 5 min), and the block was transferred to the bench top to anneal strands by gradual cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). Saccharomyces cerevisiae nucleoside diphosphate kinase (NDPK) enzyme (catalog no. N0379), adenosine diphosphate (ADP) and cobalt(II) chloride hexahydrate (catalog no. 255599) were purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 nM) into Annealing Buffer. Wash Buffer was 20 mM Tris, pH 8.0, 200 mM KCl, 200 mM potassium glutamate, 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was Wash Buffer containing low CoCl2 (0.050 mM). Reaction Buffer was Binding Buffer containing high CoCl2 (15 mM). EDTA Wash Buffer was Binding Buffer containing 1.0 mM EDTA. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP, and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), CoCl2 (100 μM) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by dNTP incorporation (dissociation phase) in Reaction Buffer containing CoCl2 (15 mM), NDPK, ADP (1 mM) and salmon sperm DNA (500 μg/mL). Biosensors were transferred to EDTA Wash Buffer followed by re-equilibration in Reaction Buffer

without enzyme, nucleotide or divalent cation. Similarly, biosensors were transferred cyclically to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) as indicated. Duplicate cycles of binding and incorporation were repeated for each dNTP to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0330]** Results of the procedure are presented in Figure 15. In assays for binding to biosensor coated with ssDNA primer-template, Klenow (exo(-)) enzyme bound poorly to the biosensor in the absence of dNTP (Figure 15, hatched bars). In the presence of the correct individual dNTPs, strong peaks were observed for the first 40 cycles (Figure 15, black bars) that correspond to the 100% correct DNA sequence for the first 30 nucleotides, assuming that each homopolymer is compressed into a single peak. Thereafter, small peak heights were observed in cycles 41 onward that did not correspond to discernable sequence. These results demonstrate the ability to sequence double-stranded DNA using Klenow exo(-) fragment of DNA polymerase with the binding of enzyme-dNTP-primer/template ternary complex in examination phase mediated by low Co2+ concentration followed by dNTP incorporation in the presence of high Co2+ concentration.

**[0331]** Example 16 describes procedures used to demonstrate extended sequence determnation. As indicated below, the results confirmed the ability to sequence single-stranded DNA using a DNA polymerase (Klenow exo(-) fragment), where the binding of enzyme-dNTP-primer/template ternary complex in the examination phase was stabilized by a divalent non-catalytic cation (i.e., Ni2+). Cognate dNTP was subsequently incorporated into the primer of the primed template nucleic acid molecule via exchange with divalent catalytic cation (i.e., MgCl2).

## Example 16

Long Read-Lengths by Sequencing Single-Stranded DNA using Nickel-Enhanced Binding, Magnesium Exchange and Catalysis in the Presence of Polymerase Trap and dNTP-Scavenging Enzyme

**[0332]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S C4493A with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGATGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:21). The DNA sequence of primer oligonucleotide 4496-4509AS was:

5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). Oligonucleotides were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides "Btn-4460-4509S C4493A" and "4496-4509AS" were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). The tube containing oligonucleotide solutions was loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Klenow (3′→5′ exo(-)) fragment of E. coli DNA polymerase was purchased from Enzymatics (Beverly, MA; catalog no. P7010-LC-L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). Saccharomyces cerevisiae nucleoside diphosphate kinase (NDPK) enzyme (catalog no. N0379), adenosine diphosphate (ADP) and nickel(II) sulfate hexahydrate (catalog no. 467901) were purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 nM) into Annealing Buffer. Wash buffer was 20 mM Tris buffer (pH 8.0), 200 mM KCl, 200 mM potassium glutamate, 0.01% (v/v), Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was Wash Buffer containing 2.0 mM Ni(II)SO4. Reaction Buffer was Binding Buffer containing MgCl2 (10 mM). EDTA Wash Buffer was Binding Buffer containing 1.0 mM EDTA. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The OCTET® QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. Biosensors were transferred to Binding Buffer containing Klenow exo(-) (68 Unit/mL), Ni(II)SO4 (2.0 mM) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by dNTP incorporation (dissociation phase) in Reaction Buffer containing MgCl2 (10 mM), NDPK, ADP (1 mM) and salmon sperm DNA (500 μg/mL). Biosensors were transferred to EDTA Wash Buffer followed by re-equilibration in Reaction Buffer without enzyme, nucleotide or divalent cation. Similarly,

biosensors were transferred cyclically to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) as indicated. Duplicate cycles of binding and incorporation were repeated for each dNTP to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0333]** Results of the procedure are shown in Figure 16. In assays for binding to biosensor coated with ssDNA primer-template, Klenow (exo(-)) enzyme bound poorly to the biosensor in the absence of dNTP (Figure 16, "Klenow"). In the presence of the correct individual dNTPs, strong peaks were observed (Figure 16, "Klenow+dNTP") that correspond to the 100% correct DNA sequence for the first 32 nucleotides (CATCAGGATGAACCGGGGCAGGGATTGCAGGC (SEQ ID NO:23)), assuming that each homopolymer is compressed into a single peak. After 750 minutes, signal was minimal and did not yield discernable sequence for the final four nucleotides (TCAC). Homopolymer compression could result from polymerase incorporating more than one dNTP in the reaction buffer. Two methods were employed to prevent homopolymer compression by conditions intended to support single-turnover incorporation. First, to block free Klenow from re-binding to the primer/template and incorporating a second dNTP, some Reaction Buffers contained an excess of salmon sperm DNA as a polymerase trap. Second, to prevent free dNTP in the Reaction Buffer from re-binding the Klenow-primer/template complex and being enzymatically incorporated into the nascent chain, Reaction Buffers containing NDPK and ADP were intended to convert free dNTP to dNDP and ATP so that the dNDP cannot be incorporated by polymerase. The ternary complex was formed in the (association phase), which was followed by dissociation in Reaction Buffers. Reaction buffers containing the polymerase trap demonstrated an accumulation on the biosensor tip as evidenced by the binding amplitude exceeding the dissociation amplitude (Figure 16, "Klenow+dNTP"). By contrast, Reaction Buffers containing NDPK and ADP produced baseline resolution following dissociation, suggesting that dissociation phase was complete (Figure 16, "NDPK+ADP+Klenow+dNTP"). These results suggest a role for dNTP in re-binding to the polymerase-primer/template complex that can result in capped, non-productive termination of sequencing. Reaction Buffers containing NDPK and ADP appear to prevent these non-productive capped products by depleting the pool of free dNTP during catalysis. Single-turnover incorporation was not achieved, since homopolymer compression was observed even in the presence of salmon sperm DNA, NDPK and ADP; the expected sequence (CATCAGGATGAACCGGGGCAGGGATTGCAGGCTCAC (SEQ ID NO:24)) was

detected as CATCAGATGACGCAGATGCAGC (SEQ ID NO:25) without sequential dinucleotides (GG, AA, CC or TT), trinucleotide (GGG) or tetranucleotide (GGGG) as shown in Figure 16.

**[0334]** Example 17 describes procedures used to demonstrate extended sequence determination using a polymerase different from the one employed in the preceding Example. As indicated below, the results confirmed the ability to sequence single-stranded DNA using Bsu Pol I (large fragment), where the binding of enzyme-dNTP-primer/template ternary complex in examination phase was stabilized by a divalent non-catalytic cation (i.e., Ni2+). Cognate dNTP was subsequently incorporated into the primer of the primed template nucleic acid molecule via exchange with divalent catalytic cation (i.e., MgCl2). Homopolymer resolution was improved by using competing dNDP in Reaction Buffers.

## Example 17

Homopolymer Resolution by Sequencing Single-Stranded DNA using Nickel(II)-Enhanced Binding, Magnesium Exchange and Catalysis in Presence of Polymerase Trap and 2'-Deoxyribonucleoside Diphosphate

**[0335]** Materials and methods used in the procedure were as follows. The DNA sequence of template oligonucleotide Btn-4460-4509S C4493A with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGATGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO:21). The DNA sequence of primer oligonucleotide 4496-4509AS was: 5'-CCCGCCATGAGCTC-3' (SEQ ID NO:10). Oligonucleotides were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotides "Btn-4460-4509S C4493A" and "4496-4509AS" were combined (10 μM each strand) in a tube containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). The tube containing oligonucleotide solutions was loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Bsu DNA polymerase I (large fragment) from Bacillus subtilis lacking exonuclease activity was purchased from New England Biolabs (Ipswich, MA; catalog no. M0330L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). Nickel(II) sulfate hexahydrate (catalog no. 467901), dCDP, dGDP and

dTDP were purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 nM) into Annealing Buffer. Wash buffer was 20 mM Tris, pH 8.0, 200 mM KCl, 200 mM potassium glutamate, 0.01% (v/v), Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was Wash Buffer containing 2.0 mM Ni(II)SO4. Reaction Buffer was 20 mM Tris buffer (pH 8.0), 50 mM KCl, MgCl2 (10 mM), 0.01% (v/v) Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. EDTA Wash Buffer was Binding Buffer containing 1.0 mM EDTA. Buffer containing Primer-Template (PT), Binding buffer containing one dNTP and Reaction Buffer were loaded (200 μL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 μL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. Biosensors were transferred to Binding Buffer containing Bsu Pol I (68 Unit/mL), Ni(II)SO4 (1.0 mM) and 100 μM dNTP (dATP, dTTP, dGTP or dCTP) as indicated (association phase) followed by dNTP incorporation (dissociation phase) in Reaction Buffer containing MgCl2 (10 mM), salmon sperm DNA (500 μg/mL) and the corresponding dNDP (except for dADP which was not used). Biosensors were transferred to EDTA Wash Buffer followed by re-equilibration in Reaction Buffer without enzyme, nucleotide or divalent cation. Similarly, biosensors were transferred cyclically to solutions containing individual deoxyribonucleoside triphosphates (dATP, dGTP, dCTP or dTTP) as indicated. Cycles of binding and incorporation were repeated for each dNTP to assess sequencing. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display.

**[0336]** Results of the procedure are presented in Figures 17A-17B. In assays for binding to biosensor coated with ssDNA primer-template, Bsu Pol I enzyme bound strongly to the biosensor in the presence of correct dNTP (Figure 17A). Signal peaks in which the Reaction Buffer contains excess dNDP (3.0 mM) correspond to correct DNA sequence of CATCAGG with the two GG of the homopolymer are resolved with detection of two distinct peaks (Figure 17A, arrows). In contrast, failure to resolve homopolymer was observed in signal peaks for control lacking dNDP in Reaction Buffer, which correspond to correct DNA

sequence of CATCAGGAT assuming that the GG homopolymer is compressed into a single G peak (Figure 17A, "Control"). The effect of dNDP on homopolymer resolution is validated by two means: (1) the height of the second G peak of the GG dinucleotide is dependent upon the concentration of dNDP in the Reaction Buffer (Figure 17A, second arrow); and (2) the next nucleotides (A and T) following the GG homopolymer are inversely related to the dNDP concentration in Reaction Buffer. Homopolymer compression could result from polymerase incorporating more than one dNTP in the reaction buffer. Two methods were employed to prevent homopolymer compression by conditions intended to support single-turnover incorporation. First, to block free Bsu Pol I from re-binding to the primer/template and incorporating a second dNTP, the Reaction Buffers contained an excess of salmon sperm DNA as a polymerase trap. Second, to prevent free dNTP in the Reaction Buffer from re-binding the Bsu Pol-primer/template complex and being enzymatically incorporated into the nascent chain, Reaction Buffers containing dNDP to compete for polymerase binding by free dNTP are expected to bind Bsu Pol-primer/template complex and block further incorporation, hence blocking homopolymer compression. The goal of single-turnover incorporation is close to being achieved, since the second peak of the GG homopolymer was 60.1% the size of the first G peak (Figure 17B), and binding for the next two nucleotides (A and T) by homopolymer compression was diminished by 73.4% and 92.0%, respectively (Figure 17B).

**[0337]** Example 18 describes the procedures used to demonstrate Kinetic resolution of homopolymer sequences. As indicated below, the results conformed the ability to quantitatively detect single and multiple incorporation into a homopolymer template using a two-step method. First, the kinetic parameters for association of the ternary complex differed for single incorporation (ALK-G1) compared to multiple incorporation (ALK-G2, ALK-G3, ALK-G4). Second, after transfer of the ternary complex-coated biosensor tip to the Reaction Buffer, the initial rate of dissociation (0-8 s) allowed incorporation of two, three or four nucleotides in a homopolymer template (ALK-G2, ALK-G3, ALK-G4) to be discerned quantitatively.

## Example 18

Kinetic Method for Homopolymer Resolution by Sequencing Single-Stranded DNA using Nickel(II)-Enhanced Binding, Magnesium Exchange and Catalysis in Presence of 2'-Deoxyribonucleoside Diphosphate and Competing Substrate

**[0338]** Materials and methods used in the procedure were as follows. The DNA sequence of wild-type ALK template oligonucleotide Btn-4460-4509S with 3' inverted dT was: Biotin-5'-GTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGAGCTCATGGCGGG-3'-(3'-dT-5') (SEQ ID NO: 7). The DNA sequence of ALK-G1 primer oligonucleotide 4494-4509AS was: 5'-CCCGCCATGAGCTCCA-3' (SEQ ID NO: 26). The DNA sequence of ALK-G2 primer oligonucleotide 4491-4509AS was: 5'-CCCGCCATGAGCTCCAGCA-3' (SEQ ID NO: 27). The DNA sequence of ALK-G3 primer oligonucleotide 4476-4509AS was: 5'-CCCGCCATGAGCTCCAGCAGGATGAACC/ideoxyI/GGGCA-3' (SEQ ID NO: 28), where "/ideoxyI/" is a 2'-deoxyinosine residue. The DNA sequence of ALK-G4 primer oligonucleotide 4482-4509AS: 5'-CCCGCCATGAGCTCCAGCAGGATGAACC-3' (SEQ ID NO: 29). Oligonucleotides were synthesized and analyzed (liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI)) by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were prepared in TE Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA) to 100 μM. To prepare the ssDNA primer/template, oligonucleotide "Btn-4460-4509S" and either "4494-4509AS," "4491-4509AS," "4476-4509AS," or "4482-4509AS" (ALK-G1, ALK-G2, ALK-G3 or ALK-G4 duplexes, respectively) were combined (10 μM each strand) in tubes containing Annealing Buffer (10 mM Tris buffer (pH 8.0), 0.1 mM EDTA, 80 mM KCl). The tubes containing oligonucleotide solutions were loaded onto a dry heat block (95°C for 5 minutes), and the block was transferred to the bench top to anneal strands by gradually cooling to ambient temperature. Bsu DNA polymerase I (large fragment) from Bacillus subtilis lacking exonuclease activity was purchased from New England Biolabs (Ipswich, MA; catalog no. M0330L). Ultra-pure bovine serum albumin (BSA) and UltraPure Salmon Sperm DNA Solution were purchased from Life Technologies (Foster City, CA). The substrate analogs 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) ("α-S-dATP"), 2'-deoxycytidine-5'-O-(1-thiotriphosphate) ("α-S-dCTP"), 2'-deoxyguanosine-5'-O-(1-thiotriphosphate) ("α-S-dGTP") and 2'-deoxythymidine-5'-O-(1-thiotriphosphate) ("α-S-dTTP") were purchased from TriLink Biotechnologies, Inc. (San Diego, CA). Nickel(II) sulfate hexahydrate (catalog no. 467901) was purchased from Sigma (St. Louis, MO). All reagents and solutions were molecular biology grade. Primer-template duplex was diluted (100 nM) into Annealing Buffer. Wash buffer was 30 mM Tris, pH 8.0, 160 mM KCl, 160 mM potassium glutamate, 0.01% (v/v), Tween-20, 100 μg/mL bovine serum albumin, 1.0 mM dithiothreitol. Binding Buffer was Wash Buffer containing Bsu Pol I (68 Unit/mL) 100 μM dGTP + 1.0 mM Ni(II)SO4. Reaction Buffer was Wash Buffer containing Bsu (1 U/mL), MgCl2 (80 μM), dGTP (28.1 μM), α-S-dGTP (162 μM). EDTA Wash Buffer was Wash

Buffer without Ni(II)SO4 but containing 1.0 mM EDTA. Buffer containing Primer-Template (PT), Binding Buffer and Reaction Buffer containing were loaded (200 µL/well) into a Greiner 96-well black microplate (Sigma-Aldrich, St. Louis, MO; catalog number M9685), and PCR-grade mineral oil (Sigma-Aldrich, St. Louis, MO; catalog no. M8662) was applied (75 µL/well). High-precision streptavidin biosensors (Pall ForteBio Corp., Menlo Park, CA; catalog number 18-5117) were re-hydrated in Annealing Buffer for approximately 10 minutes before use. The Octet QK biosensor system (Pall ForteBio Corp., Menlo Park, CA) was set for 30°C operation and was programmed to coat the biosensors with Primer-Template and wash away unbound Primer-Template with Wash Buffer. Biosensors were transferred to Binding Buffer (association phase) followed by dNTP incorporation (dissociation phase) in Reaction Buffer. Biosensors were transferred to EDTA Wash Buffer followed by re-equilibration in Reaction Buffer without enzyme, nucleotide or MgCl2. Monitoring data generated by the Octet interferometry instrument were imported into Microsoft Excel and Prism software (GraphPad Software, San Diego, CA) for display. The association phase time series were fitted to a single exponential association equation using Prism software. Association phase kinetic parameters (kobs and amplitude) were analyzed by InStat statistical software (GraphPad Software, San Diego, CA) using the Dunnett test with single incorporation of G as the control condition. The dissociation phase time series were fitted to a double exponential dissociation equation using Prism software.

**[0339]** Results of the procedure are shown in Figure 18A-18E. In assays for binding to biosensor coated with ssDNA primer-template, Bsu Pol I enzyme bound strongly to the primer/template-coated biosensor in the presence of correct dGTP (Figs. 18A and 18B). Signals for binding to ALK-G1 (Figure 18A) were higher than binding to ALK-G2, ALK-G3 and ALK-G4 (Figs. 18A and 18B). After biosensor coated with ternary complexes was transferred to incorporation buffer containing polymerase, dGTP and α-S-dGTP, Ni2+ and Mg2+, distinct dissociation time courses were observed depending on the ALK template (Figure 18C). The association phase for formation of the ternary complex was analyzed for how the association kinetic parameters may be affected by the number of nucleotides for incorporation into the homopolymer of the primer/template. Primer/templates with multiple (2-4) nucleotides for incorporation into the homopolymer had lower amplitude for association than the control single incorporation (Figure 18D), which was statistically significant ($p<0.01$) by the Dunnett test. Similarly, primer/templates with multiple (2-4) nucleotides for incorporation into the homopolymer had higher apparent kinetic constant for association (kobs) than the control single incorporation (Figure 18D), which was statistically significant

($p<0.01$) by the Dunnett test. This finding indicates that single incorporation is kinetically distinguishable from multiple incorporation in a homopolymer template. Finally, the observed dissociation rate (0-8 s following transfer into Reaction Buffer) increases with increasing numbers of nucleotides for incorporation in the rank order of ALK-G2 < ALK-G3 < ALK-G4 ~ ALK-G1, where Bsu polymerase concentrations are between 0.13 – 1 U/mL (Figure 18E).

**[0340]** Example 19 describes procedures used for optimizing discrimination between correct and incorrect nucleotides by the polymerase in the absence of chemical incorporation of any nucleotide into the primer of a primed template nucleic acid molecule. The procedure focused on titrating salts that dissolve in aqueous solution to provide monovalent cations. Binding of polymerase to a primed template nucleic acid molecule was monitored in the presence of either a cognate or non-cognate nucleotide. The procedure focused on enhancement of nucleotide discrimination under conditions that preferentially destabilized binary complex formation.

## Example 19

Enhancing Polymerase Discrimination Between Cognate and Non-Cognate Nucleotides by Preferentially Destabilizing Binary Complex Formation

**[0341]** Materials and methods used in the procedure were as follows. A FORTEBIO® (Menlo Park, CA) OCTET® instrument employing biolayer interferometry to measure binding reactions at the surface of a fiber optic tip was used in a multiwell plate format to investigate differential stability of binary and ternary complexes. Template strands biotinylated at their 5'-ends were used to immobilize the primed template nucleic acid onto fiber optic tips functionalized with streptavidin (SA) according to standard procedures. The expected sequence read from the biotinylated template DNA had a potential read length of 86 nucleotides, where the next correct nucleotide to be added to the primer was dCTP. Tips were first equilibrated in a Tris-buffered solution containing 30 mM Tris-HCl (pH 8.0), and 0.1 mM EDTA before commencing the cycling protocol. Independent binding reactions for the two test nucleotides (i.e., cognate and non-cognate nucleotides) were carried out in the presence of concentrations of NaCl, KCl, or potassium glutamate that varied from 50 mM to 500 mM. A fourth trial was conducted using a fixed 160 mM concentration of potassium glutamate, while the concentration of KCl varied from 50 mM to 500 mM. In all instances, the reaction mixture used during the examination step contained Tris-HCl (pH 8.0), 0.01%

Tween-20, 100 µg/ml BSA, 2 mM NiSO4, 350 U/ml Bsu DNA polymerase large fragment; and one of the nucleotides at a concentration of 100 µM (dCTP was used as a cognate nucleotide, and dGTP was used as a non-cognate nucleotide). Following each examination step, tips were exposed to a buffer containing 30 mM Tris-HCl (pH 8.0), 500 mM KCl, 2 mM EDTA and 0.05% Tween-20 for 20 seconds to strip enzyme complexes from the primed template nucleic acid. The stripping step was followed by a 15 second exposure to examination buffer without enzyme, dNTP or divalent cations to regenerate tips for the next cycle of examination. When using a single contacting step to effect binding of polymerase and nucleotide to the primed template nucleic acid, the binding step was 45 seconds long, with binding interactions being monitored continuously. This was accomplished by contacting the primed template nucleic acid with a single solution that included the polymerase and test nucleotide. Results from interferometry monitoring were analyzed to identify formation of ternary complexes (i.e., identifying cognate nucleotide) or binary complexes (i.e., identifying non-cognate nucleotide). Numerical results from the interferometry testing are presented in Tables 5-8. Some of the rounded binary complex measurement values were so low that they appeared as 0.00 in the table, yet permitted calculation of a fold enhancement.

**Table 5**
Establishing Conditions for Optimal Discrimination in the Sequencing-by-Binding Procedure

| KCl conc (mM) | Ternary Complex Signal (Δλ, nm) | Binary Complex Signal (Δλ, nm) | Fold Enhancement (Ternary/Binary) |
|---|---|---|---|
| 50 | 2.98 | 2.90 | 1.03 |
| 75 | 2.59 | 2.68 | 0.97 |
| 100 | 2.29 | 2.29 | 1.00 |
| 125 | 1.93 | 1.98 | 0.98 |
| 150 | 1.67 | 1.67 | 1.00 |
| 175 | 1.41 | 1.43 | 0.99 |
| 200 | 1.30 | 1.20 | 1.08 |
| 225 | 1.20 | 1.03 | 1.17 |
| 250 | 1.11 | 0.82 | 1.35 |
| 275 | 1.02 | 0.67 | 1.51 |
| 300 | 0.96 | 0.47 | 2.04 |
| 325 | 0.71 | 0.36 | 1.97 |
| 350 | 0.64 | 0.17 | 3.72 |
| 375 | 0.49 | 0.15 | 3.21 |
| 400 | 0.39 | 0.04 | 9.48 |
| 425 | 0.28 | 0.00 | 85.77 |
| 450 | 0.19 | 0.01 | 16.47 |
| 475 | 0.14 | 0.03 | 4.89 |
| 500 | 0.09 | 0.04 | 2.69 |

**Table 6**
Establishing Conditions for Optimal Discrimination in the Sequencing-by-Binding Procedure

| NaCl conc (mM) | Ternary Complex Signal (Δλ, nm) | Binary Complex Signal (Δλ, nm) | Fold Enhancement (Ternary/Binary) |
|---|---|---|---|
| 50 | 3.33 | 3.11 | 1.07 |
| 75 | 2.77 | 2.51 | 1.10 |
| 100 | 2.34 | 2.17 | 1.07 |
| 125 | 1.99 | 1.91 | 1.04 |
| 150 | 1.73 | 1.67 | 1.04 |
| 175 | 1.51 | 1.36 | 1.11 |
| 200 | 1.36 | 1.15 | 1.19 |
| 225 | 1.28 | 1.01 | 1.27 |
| 250 | 1.18 | 0.80 | 1.47 |
| 275 | 1.11 | 0.67 | 1.65 |
| 300 | 0.99 | 0.53 | 1.86 |
| 325 | 0.93 | 0.34 | 2.76 |
| 350 | 0.74 | 0.25 | 2.94 |
| 375 | 0.61 | 0.17 | 3.62 |
| 400 | 0.48 | 0.08 | 5.93 |
| 425 | 0.38 | 0.07 | 5.16 |
| 450 | 0.26 | 0.04 | 6.65 |
| 475 | 0.19 | 0.02 | 10.28 |
| 500 | 0.21 | 0.00 | ND |

**Table 7**
Establishing Conditions for Optimal Discrimination in the Sequencing-by-Binding Procedure

| Potassium Glutamate (mM) | Ternary Complex Signal (Δλ, nm) | Binary Complex Signal (Δλ, nm) | Fold Enhancement (Ternary/Binary) |
|---|---|---|---|
| 50 | 1.67 | 1.59 | 1.05 |
| 75 | 1.69 | 1.68 | 1.01 |
| 100 | 1.67 | 1.64 | 1.02 |
| 125 | 1.65 | 1.57 | 1.05 |
| 150 | 1.62 | 1.51 | 1.07 |
| 175 | 1.56 | 1.44 | 1.08 |
| 200 | 1.51 | 1.40 | 1.07 |
| 225 | 1.53 | 1.38 | 1.11 |
| 250 | 1.48 | 1.27 | 1.17 |
| 275 | 1.52 | 1.24 | 1.22 |
| 300 | 1.50 | 1.23 | 1.23 |
| 325 | 1.46 | 1.15 | 1.27 |
| 350 | 1.39 | 1.07 | 1.30 |
| 375 | 1.36 | 1.03 | 1.32 |
| 400 | 1.36 | 0.99 | 1.36 |
| 425 | 1.33 | 0.91 | 1.46 |
| 450 | 1.31 | 0.86 | 1.52 |
| 475 | 1.28 | 0.82 | 1.56 |
| 500 | 1.28 | 0.80 | 1.60 |

**Table 8**
Establishing Conditions for Optimal Discrimination in the Sequencing-by-Binding Procedure

| 160 mM Potassium Glutamate / KCl conc (mM) | Ternary Complex Signal (Δλ, nm) | Binary Complex Signal (Δλ, nm) | Fold Enhancement (Ternary/Binary) |
|---|---|---|---|
| 50 | 1.57 | 1.27 | 1.24 |
| 75 | 1.49 | 1.06 | 1.41 |
| 100 | 1.52 | 0.89 | 1.71 |
| 125 | 1.44 | 0.70 | 2.05 |
| 150 | 1.40 | 0.57 | 2.46 |
| 175 | 1.30 | 0.39 | 3.31 |
| 200 | 1.19 | 0.29 | 4.15 |
| 225 | 1.12 | 0.21 | 5.35 |
| 250 | 0.98 | 0.14 | 7.02 |
| 275 | 0.88 | 0.10 | 9.07 |
| 300 | 0.76 | 0.09 | 8.34 |
| 325 | 0.65 | 0.07 | 8.97 |
| 350 | 0.54 | 0.07 | 7.94 |
| 375 | 0.45 | 0.04 | 12.46 |
| 400 | 0.37 | 0.03 | 13.75 |
| 425 | 0.31 | 0.02 | 13.28 |
| 450 | 0.25 | 0.03 | 7.24 |
| 475 | 0.22 | 0.05 | 4.54 |
| 500 | 0.21 | 0.07 | 2.97 |

**[0342]** Results presented in Tables 5-8 showed how salts that provide monovalent cations preferentially destabilized binary complex formation, and enhanced polymerase discriminatory potential under conditions that precluded nucleotide incorporation. Substantially all of the monovalent cation and glutamate ion in the binding reaction mixtures were provided by the added salts, and so contributions of monovalent cations from buffer and other sources were regarded as insignificant for this analysis. Binding signals were measured and compared for polymerase interaction with a primed template nucleic acid in the presence of cognate or non-cognate nucleotides. Again, these results apply to binding reactions that discriminate between correct and incorrect nucleotides without relying on nucleotide incorporation as a readout. Of course, nucleotide used in the procedure can be labeled or unlabeled. Preferably however, the nucleotide is a native nucleotide that is unlabeled.

**[0343]** Binary complex formation was destabilized under conditions used in the examining step. Dose-response titrations with model salts that provide monovalent cations (e.g., NaCl,

KCl, and potassium glutamate) similarly indicated that binary and ternary complexes were differentially sensitive to the added salt. As the salt concentration increased, binary complexes became progressively less stable relative to the corresponding ternary complexes. An example concentration range for enhancing discrimination between the ternary complex (indicating the presence of cognate nucleotide) and binary complex (indicating the presence of non-cognate nucleotide) was 200 mM to 500 mM of the salts providing monovalent cations. These values were useful in procedures wherein polymerase binding to the primed template nucleic acid molecule served as an indicator of nucleotide identity (i.e., cognate versus non-cognate). Dose-response titrations of potassium glutamate similarly indicated differential stability of the binary and ternary complexes. Here again the binary complex became progressively less stable relative to the corresponding ternary complex as the concentration of added glutamate salt increased. Since potassium glutamate includes two potassium ions for each glutamate ion, the contribution to the potassium ion concentration in the reaction mixture was double the concentration of added glutamate salt.

**[0344]** Under the non-incorporating conditions of the described procedure, and in the absence of a source of glutamate, concentrations of salts containing monovalent cations that were below 200 mM provided modest discrimination between cognate and non-cognate nucleotide binding. However, the polymerase enzyme under these conditions maintained a high binding capacity. Titration of either of KCl or NaCl alone showed destabilization of binary complex formation, and that fold-discrimination was achieved at concentrations above 250 mM, although there was some loss of polymerase binding activity. Potassium glutamate also destabilized binary complex formation, and enhanced discriminatory potential substantially over the full range of concentrations that were tested. To obtain long read lengths using the sequencing-by-binding procedure, high signals that discriminate correct base calls from incorrect base calls must be maintained over many bases of the targeted template. In the interest of maintaining a high binding capacity with improved fold-discrimination, constant levels of glutamate (e.g., 0 mM, 80 mM, 160 mM, and 320 mM) were used while titrating KCl from 50 mM to 500 mM.

**[0345]** Enhancement of the discriminatory potential of the polymerase resulted from combining a salt that provided monovalent cations together with a glutamate salt under conditions where polymerization was precluded. For example, at either 150 mM of KCl or 150 mM of potassium glutamate there was modest discrimination between formation of ternary and binary complexes. However, combining the two agents enhanced the discriminatory potential. The effect could not be attributed to either the increased

monovalent cation concentration or the glutamate ion concentration alone. Preferred conditions for destabilizing binary complex formation during the examination step included a salt providing monovalent cations, where the concentration of the salt fell in the range of from 50 mM to 1,500 mM, more preferably in the range of from 50 mM to 500 mM.

**[0346]** Additional procedures consistent with the foregoing description, together with further analysis of results from Tables 5-8, were carried out to better establish conditions useful for destabilizing binary complexes in the examination step. Titration with a salt that provided monovalent metal cations (e.g., KCl) yielded a plot showing that a maximum difference between signals for ternary and binary complexes (a parameter relevant to the sequencing-by-binding procedure) occurred when the salt was in the concentration range of from about 300 mM – 350 mM (see Table 5). However, a shift toward lower concentration ranges was achieved when a glutamate salt (e.g., potassium glutamate) was also included. Here the difference between signals was most pronounced in the range of from about 100 mM – 300 mM of the salt providing monovalent cations (e.g., monovalent metal cations) when a glutamate salt was included at a concentration of from 80 mM – 320 mM. Indeed, the difference between signals was most pronounced in the range of from: 100 mM – 200 mM at 320 mM of the glutamate salt; 150 mM – 250 mM at 160 mM of the glutamate salt (see Table 8); and 175 mM – 300 mM at 80 mM of the glutamate salt. Maximum signal ratios for ternary complexes over binary complexes (a different parameter measuring discriminatory effectiveness) centered at about 450 mM for the salt providing monovalent metal cations when the titration was carried out in the absence of the glutamate salt. Ratios in the combined titration were most pronounced when the salt providing monovalent metal cations fell in the range of from: 200 mM – 250 mM at 320 mM of the glutamate salt; 350 mM – 450 mM at 160 mM of the glutamate salt; and 275 mM – 350 mM at 80 mM of the glutamate salt. In all instances, the concentration of salt providing monovalent cations that produced maximum discriminatory ratios was substantially higher than the concentration needed to achieve the maximum differences between the signals indicating ternary and binary complexes. Notably, testing conducted using the glutamate salt alone (i.e., without added KCl) at substantially the concentrations cited above (i.e., 75 mM, 150 mM, and 325 mM) showed either substantially no enhancement of discriminatory potential, or only very modest enhancement (see Table 7). In the absence of added salt, concentrations of the glutamate salt required for generating useful conditions in the examination step preferably were higher than 325 mM.

**[0347]** Still further testing conducted using the Bst and Klenow polymerases confirmed that binary complex formation was preferentially destabilized by examination conditions that included a glutamate salt and/or salts that provided monovalent cations (e.g., monovalent metal cations). Generally speaking, as the concentration of potassium glutamate increased, the concentration of KCl that destabilized binary complexes could be decreased while still giving good discriminatory results. When the potassium glutamate concentration was as high as 500 mM, added KCl could be omitted completely (i.e., the concentration of added KCl was 0 mM) while still providing good discrimination between correct and incorrect nucleotides. Likewise, when the potassium glutamate concentration was 320 mM, a KCl concentration of 25 mM gave outstanding results using the Klenow polymerase. When using the Bst enzyme, somewhat higher concentrations of KCl were required to achieve the best results.

**[0348]** Thus, while optimal discriminatory conditions differed somewhat between the different polymerases, it was generally true that salts providing monovalent cations (e.g., monovalent metal cations), either alone or in combination with another glutamate salt, preferentially destabilized binary complexes, thereby enhancing discrimination between correct and incorrect nucleotide binding. It was even demonstrated that the glutamate salt could serve as the salt providing the monovalent metal cations.

**[0349]** Based on the foregoing, and further in view of the additional testing according to the procedures described in the Example, preferred conditions for destabilizing binary complex formation were as follows. The reaction mixture employed in the examination step should contain a source of monovalent cations, preferably monovalent metal cations (e.g., metal cations having oxidation state of +1). This conveniently can be accomplished by including in the reaction mixture a salt that provides monovalent cations, where the salt is included at a concentration of from about 50 mM – 1,500 mM. Preferably, the salt is included at a concentration of from about 50 mM – 500 mM. Still more preferably, the salt is included at a concentration of from about 100 mM – 300 mM. Optionally, the salt that provides monovalent cations also provides glutamate anions (i.e., the salt is a glutamate salt). When the salt used is these concentration ranges is a salt other than a glutamate salt, the reaction mixture containing the salt optionally may further include a glutamate salt at a concentration of from about 10 mM – 1,600 mM, more preferably in the range of from 10 mM – 500 mM, or still more preferably in the range of from 80 mM – 320 mM. Generally speaking, reaction conditions used for contacting primed template nucleic acid molecules with the reaction mixture in an examination step preferably favor ternary complex formation over binary

complex formation by at least two-fold, by at least five-fold, or even more. Notably, reduced ternary complex formation can be accommodated in the examination step of the sequencing-by-binding procedure because an incorporation reaction can be conducted with high efficiency under different reaction conditions that promote interactions (e.g., including interactions that permit or favor formation of binary complexes) between polymerase and the primed template nucleic acid molecule.

**[0350]** Yet another approach for reducing or minimizing contributions due to signals resulting from binary complex formation involves the reagent and approach used for delivering the polymerase to the primed template nucleic acid molecules. Here a specialized "polymerase delivery reagent" (PDR) can be used for sequencing-by-binding procedures, subject to certain restrictions on the nucleotide undergoing examination. The PDR advantageously helps minimize unwanted binary complex formation, while still permitting efficient formation of ternary complexes. The disclosed PDR can be used on a variety of platforms (e.g., label-free SPR; fluorescent pols in flow cells; etc.) to obtain improved results. Generally speaking, signal-to-noise ratios are improved in procedures that employ the PDR and monitor polymerase binding as indicators of cognate and non-cognate nucleotide identity, and this aids in correct base calling and extended reads.

**[0351]** Prior to development of the PDR, polymerase reagent was delivered to immobilized primed template nucleic acid molecules in the absence of free nucleic acid molecules. At least some level of background signal always was detected due to binding of the polymerase to primed template nucleic acid molecules in the absence of cognate nucleotide. Indeed, conditions could be identified where background binding was so extensive that no observable difference was detected when the correct base was added. This background signal was attributed to binary complex formation, where polymerase bound to primed template nucleic acid molecules in the absence of cognate nucleotide.

**[0352]** As described elsewhere herein, alternative approaches used to reduce signal arising from binary complex formation focused on concentrations and combinations of salts. For example, concentration ranges of KCl and potassium glutamate were shown useful and adequate for suppressing binary complex formation. However, alternative use of the PDR advantageously permits salt conditions used for optimizing reduction of binary complex formation to be changed – still with outstanding results. This further illustrated that multiple different approaches can be used for suppressing or "destabilizing" binary complex formation.

**[0353]** Example 20 describes procedures that demonstrated how use of the PDR advantageously reduced signal arising from binary complex formation while permitting correct ternary complex formation. The polymerase delivery reagent included: (a) a DNA polymerase; and (b) a primed template nucleic acid. The included primed template nucleic acid is free in solution, and can interact with the polymerase to form a complex (e.g., a binary complex).

**Example 20**

Polymerase Delivery Reagent (PDR) for Reducing Binary Complex Formation in a Sequencing-by-Binding Procedure

**[0354]** Four streptavidin-coated Octet tips were contacted with binding buffer solutions containing biotinylated template DNA strands hybridized to primers to prepare immobilized primed template nucleic acid molecules. In this procedure, binding buffer was an ACES-buffered (pH 7.5) solution that included KCl, potassium glutamate, TbCl3, Tween-80 and BSA. Thereafter the biosensor tips were washed using binding buffer that did not include nucleic acids. Binary complex formation was accomplished by contacting the biosensor tips with binding buffer containing 1 μM of a Bst DNA polymerase engineered to contain an added cysteine residue together with either 0 nM, 10 nM, 100 nM or 1,000 nM of a soluble or solution-phase (i.e., free in solution) primed template nucleic acid that was not biotinylated. The solution used to form binary complexes representing background signal did not include the cognate nucleotide. Notably, the solution-phase primed template nucleic acid had a sequence different from the sequence of the primed template nucleic acids immobilized to the biosensor tip. Finally, ternary complexes were formed by contacting the biosensor tip with a binding buffer solution that included either 0 nM, 10 nM, 100 nM or 1,000 nM of a soluble or solution-phase (i.e., free in solution) primed template nucleic acid together with 100 μM of the nucleotide that was the next correct nucleotide for the immobilized primed template nucleic acid molecule. The solution-phase nucleotide included in the binding buffer was not the cognate nucleotide for the solution-phase primed template nucleic acid. When using different reagents containing solution-phase nucleotides to be tested as cognate nucleotides for immobilized nucleic acid features (e.g., primed template nucleic acids immobilized directly or indirectly to a surface within a flow cell), the solution-phase nucleotide should not be the cognate nucleotide for the solution-phase primed template nucleic acid. In this way,

only binary complexes, and not ternary complexes, can form between the solution-phase nucleotide and the solution-phase primed template nucleic acid molecule. Optionally, there can be more than one solution-phase primed template nucleic acid molecule in the reagent solution containing the solution-phase nucleotide. More particularly, there can be up to three different solution-phase primed template nucleic acids in a single reagent solution containing the solution-phase nucleotide, where the solution-phase nucleotide is not the cognate nucleotide for any of the solution-phase primed template nucleic acids.

**[0355]** Results from these procedures, shown in Figure 19, confirmed that delivery of the polymerase in combination with a solution-phase nucleotide and a solution-phase primed template nucleic acid, where the solution-phase nucleotide was not the cognate nucleotide for the solution-phase primed template nucleic acid advantageously suppressed or destabilized binary complex formation while still permitting ternary complex formation. More particularly, as the concentration of the solution-phase primed template nucleic acid increased, there was a corresponding decrease in the magnitude of the polymerase binding signal in the absence of nucleotide. Here only binary complex formation was possibly. Thereafter, inclusion of nucleotide that was the cognate nucleotide for the primed template nucleic acid immobilized to the biosensor tip gave increased binding signals. Comparison of the results from this procedure indicated that an optimal difference in that signal magnitudes occurred when the solution-phase primed template nucleic acid was present at the 100 nM concentration (see Figure 20). Accordingly, routine procedures can be followed to optimize concentrations of the solution-phase primed template nucleic acid needed to give good results.

**[0356]** Taken together, these results show how use of the PDR reagent improved discrimination between binary and ternary complex formation in a way that was not previously possible. Similarly good results were obtained using two independent DNA polymerase enzymes. This confirmed the general utility of the approach.

**[0357]** In some embodiments, a plurality of independent examination reactions is completed before performance of any incorporation reaction. According to different preferred approaches, this can involve carrying out examination reactions using a detectably labeled polymerase, or using one or more detectably labeled nucleotides.

**[0358]** In one approach, examination reactions can be carried out using as few as a single labeled polymerase, rather than a collection of distinguishably labeled polymerases. Primed template nucleic acids (e.g., a collection of spaced apart features, such as immobilized RCA products or beads displaying primed template nucleic acids) can be contacted with

combinations of the labeled polymerase and one or more nucleotides. Following a period to allow binding, and optionally a wash step to remove any non-complexed materials (e.g., labeled polymerase) from the binding reaction mixture, interaction of the labeled polymerase with the primed template nucleic acid molecule is assessed. After completion of a first examination reaction, and removal or stripping of the polymerase-nucleotide(s) combination from the primed template nucleic acid molecule, a second examination reaction is carried out by contacting the primed template nucleic acid molecule with a second polymerase-nucleotide(s) combination. The detection and removal procedures are then repeated. Nucleotides used in this procedure need not carry any detectable label, because it is the localization of the polymerase to the primed template nucleic acid molecule that is detected or monitored in this procedure as an indicator of cognate nucleotide identity. Again, the detectably labeled polymerase preferentially produces a signal that does not substantially change as the result of interaction with any nucleotide. More particularly, the signal is substantially uniform. By this approach, localization of the signal, as contrasted with production of the signal, can be monitored to assess ternary complex formation. Procedures can be carried out using either non-catalytic metal ions or reversibly blocked primers in the primed template nucleic acid molecules to stabilize ternary complexes. Thus, serial examination reactions can be carried out using as few as a single type of detectably labeled polymerase in a procedure that repeatedly or iteratively tests different nucleotides or nucleotide combinations for the ability to promote ternary complex formation.

**[0359]** In an alternative approach, detectably labeled nucleotides can be used in place of detectably labeled polymerase(s). Optionally, the labeled nucleotides harbor fluorescent moieties, Raman-active moieties, *etc.* Optionally, each different labeled nucleotide among a plurality of labeled nucleotides used in a procedure includes the same type of fluorescent moiety. Alternatively, each different labeled nucleotide among a plurality of labeled nucleotides used in a procedure can include different fluorescent moieties that are not distinguished from each other by their optical properties during a procedure. For example, a single or common detection channel or wavelength range can be used for detecting the different labeled nucleotides in ternary complexes. Preferably, optical properties (*e.g.*, excitation or emission spectra) of fluorescent moieties of the labeled nucleotides remain substantially unchanged when the labeled nucleotide are free in solution (*i.e.*, not included in a ternary complex) or participating in a ternary complex. Thus, labeled nucleotides need not be labeled with any conformationally sensitive label, or intercalating dye that emits a distinctive optical signal when the nucleotide participates in a ternary complex. As well,

success of the technique does not require that the detectable labels participate in energy transfer relationships with any other fluorescent dye or quencher moieties (*e.g.*, detectable moieties preferably need not be FRET partners). In some embodiments, polymerase-nucleotide combinations can be delivered to a primed template nucleic acid molecule (*e.g.*, a primed template nucleic acid molecule immobilized within a flow cell) in serial fashion, one at a time. Cognate nucleotide identification can involve detecting a ternary complex by detecting the label moiety attached to the nucleotide. For example, this can involve associating cognate nucleotide identity with the identity of the nucleotide that was contacted to the primed template nucleic acid molecule in combination with the polymerase, where a ternary complex was formed as a consequence. Simply detecting the ternary complex where polymerase-nucleotide combinations were delivered to the primed template nucleic acid in a known order (*e.g.*, in a serial fashion) can be sufficient to identify the next correct nucleotide.

**[0360]** In some preferred approaches, steps that involve contacting a polymerase in combination with one or more nucleotides to a primed template nucleic acid molecule (*e.g.*, an immobilized primed template nucleic acid molecule) can be carried out using a blocked primed template nucleic acid molecule in the presence of a catalytic metal ion (*e.g.*, magnesium ion or manganese ion). The blocked primed template nucleic acid molecule can include a primer with a 3' terminal nucleotide having a reversible terminator moiety, as described herein. Practically speaking, reagents or solutions containing the different polymerase-nucleotide combinations can also include the catalytic metal ion. When contacted to the blocked primed template nucleic acid molecule, incorporation of cognate nucleotide is precluded by the presence of a reversible terminator moiety on the 3' terminal nucleotide of the primer strand hybridized to the template strand of the primed template nucleic acid molecule. Ternary complexes including the blocked primed template nucleic acid molecule can form efficiently without incorporation, regardless of the catalytic capacity of the polymerase. While not wishing to be constrained by any particular theory of operation, one possibility is that the presence of the catalytic metal ion helps maintain integrity of the polymerase and its ability to recognize and discriminate between cognate and non-cognate nucleotides. Alternatively, inclusion of the catalytic metal ion may beneficially impact the structure of the ternary complex itself. Notwithstanding the underlying mechanism, some preferred approaches for carrying out the disclosed techniques can involve contacting a blocked primed template nucleic acid molecule (*e.g.*, having a primer with a reversible terminator moiety attached to the 3' terminal nucleotide) with a polymerase-nucleotide combination in the presence of a catalytic metal ion (*e.g.*, either or both of magnesium ion

and manganese ion). Preferably, the contacting step is carried out in the presence of the catalytic metal ion, and in the absence of non-catalytic metal ions that inhibit nucleotide incorporation.

**[0361]** Figure 21 illustrates a simplified workflow for the iterative sequencing procedure employing a single type of detectably labeled polymerase; and Figure 22 illustrates results obtained using that procedure and protocols essentially as described herein. Here, the labeled polymerase and one nucleotide at a time contacted beads harboring a reversibly blocked primed template nucleic acid for the Alk-C2 target sequence, where the expected sequence of nucleotides was CGGG. All binding reactions were carried out in the presence of Mg2+ ion, although inclusion of this catalytic metal ion was optional. In each cycle of examining four different nucleotides in a serial fashion, the correct nucleotide was associated with the highest binding signal. This indicated that detectably labeled polymerase associated with the blocked primed template nucleic acid molecule to form ternary complexes in the expected fashion.

**[0362]** The following Example illustrates one embodiment of the sequencing-by-binding technique, where a single type of detectably labeled polymerase was used to assess binding of native nucleotides.

## Example 21

Labeled Polymerase Sequencing

**[0363]** Flow cells were prepared using magnetic 1 μM microbeads displaying synthetic primed template nucleic acids of known sequence. Briefly, streptavidin-coated MyOne C1 magnetic beads (ThermoFisher Scientific; Waltham, MA) were functionalized with a TCO-PEG4-NHS (transcyclooctene-polyethylene glycol-N-hydroxysuccinimide) moiety that reacts with free amine moieties on the streptavidin. The TCO-modified beads were then incubated in a solution containing the desired primed template nucleic acid molecule at a concentration of 100 nM. The beads were next introduced into a flow cell constructed with an aminosilane-coated coverslip that had been modified with an NHS-tetrazine ester reagent to covalently bind the TCO modified beads. The beads were allowed to settle to the surface and bind for about 15 minutes, and the bead density checked by optical microscopy. If higher bead density was required, more beads were flowed in and allowed to bind. Contents of the flow cell were "blocked" with SuperBlock (ThermoFisher Scientific) to minimize non-specific binding of reagents to the beads or background surfaces.

**[0364]** Prior to initiating the sequencing run, reagents were loaded into 15 mL conical tubes and connected to a fluidic manifold with reagent lines leading to the flow cell. The flow cell containing the bead array was mounted on a microscope equipped with a 20X objective, and then connected to the fluidic manifold. The flow cell was purged with wash reagent to equilibrate the beads and primed template nucleic acid with the starting reaction conditions. Sequencing was initiated using an automated protocol to control the order and timing of reagent delivery. Figure 21 shows a flow diagram outlining an example workflow. In this procedure labeled polymerase and a single nucleotide at a time were contacted to the immobilized primed template nucleic acid molecules. The polymerase used in the procedure was a BSU polymerase engineered to contain a cysteine that was chemically attached to a fluorescent Cy5 label.

**[0365]** Figure 22 shows the resulting maximum fluorescence intensities for equilibrium binding of fluorescently labeled polymerase, where the polymerase bound primed template nucleic acid molecules in combination with one native nucleotide at a time. Again, there was no energy transfer between the fluorescent moiety and the nucleotide to make the detection. As well, the label on the polymerase served only to provide a way to track location of the polymerase, where fluorescence of the polymerase remained substantially unchanged as a consequence of different nucleotides being present in the reaction mixtures. Maximum binding signals for each cycle were considered the correct base calls. Base calls for each cycle of the feature represented in the figure corresponded to the first four bases of one of the Alk gene fragments included in the template panel. In each case, the feature being sequenced showed a unique response to each examination step. This demonstrated how repetitive cycles of examining as few as one nucleotide at a time using a fluorescently labeled polymerase could be used for sequencing a template nucleic acid.

**[0366]** Disclosed above are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. It is to be understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed, and that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset

or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure, including steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

**[0367]** Publications cited herein, and the material for which they are cited, are hereby specifically incorporated by reference in their entireties. All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

**[0368]** It is to be understood that the headings used herein are for organizational purposes only and are not meant to limit the description or claims.

**[0369]** A number of embodiments have been described. Nevertheless, it will be understood that various modifications may be made. Accordingly, other embodiments are within the scope of the claims.

**WHAT IS CLAIMED IS:**

1. A method of determining the identity of the next correct nucleotide for a primed template nucleic acid molecule, the method comprising:

(a) providing a template nucleic acid molecule primed with a primer;

(b) serially contacting the primed template nucleic acid molecule with a plurality of reaction mixtures, each of the reaction mixtures comprising a polymerase and a different combination of two or three different test nucleotides, under conditions that stabilize ternary complexes comprising the primed template nucleic acid molecule, the polymerase and a next correct nucleotide, while precluding incorporation of any nucleotide into the primer;

(c) detecting interaction of the polymerase with the primed template nucleic acid molecule without chemical incorporation of any nucleotide into the primer of the primed template nucleic acid molecule, to determine whether ternary complexes form; and

(d) determining whether a test nucleotide that is common to at least two of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction.

2. The method of claim 1, wherein step (d) comprises determining whether a test nucleotide that is common to two of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction.

3. The method of claim 2, wherein four different reaction mixtures are serially contacted with the primed template nucleic acid molecule in step (b), wherein in aggregate each different nucleotide is present in two reaction mixtures.

4. The method of claim 1, wherein step (d) comprises determining whether a test nucleotide that is common to three of the reaction mixtures is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction.

5. The method of claim 4, wherein six different reaction mixtures are serially contacted with the primed template nucleic acid molecule in step (b), wherein in aggregate each different nucleotide is present in three reaction mixtures.

6. The method of claim 1, wherein the conditions also destabilize binary complexes comprising the primed template nucleic acid molecule and the polymerase but not the next correct nucleotide.

7. The method of claim 1, wherein the ternary complex is stabilized by the presence of a reversible terminator moiety on the 3' terminal nucleotide of the primer.

8. The method of claim 7, further comprising, after step (c), removing the reversible terminator moiety on the 3' terminal nucleotide of the primer.

9. The method of claim 1, wherein each of the test nucleotides is an unlabeled nucleotide.

10. The method of claim 9, wherein the polymerase comprises an exogenous label that is detected in step (c).

11. The method of claim 1, wherein the next correct nucleotide comprises an exogenous label that is detected in step (c).

12. The method of claim 1, further comprising (e) incorporating a nucleotide at the 3' end of the primer after step (c).

13. The method of claim 12, wherein the nucleotide that is incorporated is an unlabeled nucleotide.

14. The method of claim 12, wherein the nucleotide that is incorporated is an unlabeled reversible terminator nucleotide.

15. The method of claim 12, further comprising repeating steps (b) through (e) to sequence the primed template nucleic acid molecule.

16. A method of sequencing a primed template nucleic acid, comprising:

(a) contacting a primed template nucleic acid with a polymerase and a first combination of two or three types of test nucleotides under conditions that form a stabilized

ternary complex between the polymerase, primed template nucleic acid and a test nucleotide that is complementary to the next base of the primed template nucleic acid;

(b) detecting the ternary complex while precluding incorporation of test nucleotides into the primer;

(c) repeating steps (a) and (b) using the primed template nucleic acid, a polymerase and a second combination of two or three types of test nucleotides, wherein the second combination is different from the first combination;

(d) incorporating into the primer, after step (c), a nucleotide that is complimentary to the next base; and

(e) repeating steps (a) through (d) to identify a sequence of the primed template nucleic acid.

17. The method of claim 16, wherein the first combination comprises two types of test nucleotides.

18. The method of claim 17, wherein the second combination comprises two types of test nucleotides.

19. The method of claim 18, wherein steps (a) and (b) are carried out serially for four different combinations of two types of test nucleotides, wherein each different nucleotide type is contacted with the primed template nucleic acid two times in aggregate.

20. The method of claim 18, wherein steps (a) and (b) are carried out serially for six different combinations of two types of test nucleotides, wherein each different nucleotide type is present three times in aggregate.

21. A method of identifying the next correct nucleotide for a primed template nucleic acid molecule, said method comprising the steps of:

(a) providing a template nucleic acid molecule primed with a primer, said primer comprising a reversible terminator moiety attached to the 3' terminal nucleotide thereof;

(b) serially contacting the primed template nucleic acid molecule with a plurality of polymerase-nucleotide combinations in the presence of a catalytic metal ion, without incorporation,

wherein each of said combinations comprises a polymerase and a different nucleotide, and

whereby there is formed a ternary complex comprising the polymerase, one of the different nucleotides delivered in combination with the polymerase, and the primed template nucleic acid molecule when said one of the different nucleotides is the next correct nucleotide; and

(c) detecting the ternary complex, thereby identifying the next correct nucleotide for the primed template nucleic acid molecule as said one of the different nucleotides that contacted the primed template nucleic acid molecule in combination with the polymerase to form the ternary complex.

22. The method of claim 21, wherein step (b) comprises serially contacting the primed template nucleic acid molecule with the plurality of polymerase-nucleotide combinations in the presence of the catalytic metal ion and in the absence of any non-catalytic ion that inhibits incorporation.

23. The method of claim 22, wherein the catalytic metal ion is magnesium ion.

24. The method of claim 22, wherein the polymerase is a labeled polymerase comprising an exogenous detectable label that produces a signal, wherein the signal produced by the exogenous detectable label of the labeled polymerase does not substantially change in the presence or absence of any nucleotide, and wherein step (c) comprises detecting the ternary complex by detecting the exogenous detectable label of the labeled polymerase.

25. The method of claim 24, wherein each of the different nucleotides of said combinations in step (b) is a different native nucleotide.

26. The method of claim 22, wherein each different nucleotide of said combination is a different labeled nucleotide comprising an exogenous detectable label that produces a signal, and wherein the signal produced by the different labeled nucleotides is substantially the same before and after formation of the ternary complex.

27. The method of claim 22, wherein the primed template nucleic acid molecule is contained within a flow cell, and wherein step (b) comprises contacting the primed template

nucleic acid molecule by flowing through the flow cell, one at a time, a plurality of reagent solutions, each comprising one of the plurality of polymerase-nucleotide combinations.

28. The method of claim 27, wherein the primed template nucleic acid molecule is disposed on a bead contained within the flow cell.

29. The method of claim 27, further comprising the step of flowing through the flow cell, between flows of each of the plurality of reagent solutions, a wash buffer that removes any of the polymerase and the different nucleotides that did not combine with the primed template nucleic acid molecule to form the ternary complex.

30. The method of claim 27, further comprising after step (c), the step of (d) flowing through the flow cell a stripping buffer that dissociates the ternary complex to remove the polymerase and said one of the different nucleotides from the primed template nucleic acid molecule.

31. The method of claim 30, further comprising removing the reversible terminator moiety attached to the 3' terminal nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule comprising an extendable primer.

32. The method of claim 31, further comprising the step of (e) incorporating a nucleotide into the extendable primer.

33. The method of claim 32, wherein step (e) comprises incorporating with a polymerase different from the polymerase of step (a).

34. The method of claim 32, wherein the nucleotide incorporated into the extendable primer comprises a reversible terminator moiety, and wherein the incorporation produces a blocked primed template nucleic acid molecule.

35. The method of claim 21, wherein the polymerase is a labeled polymerase comprising an exogenous detectable label.

36. The method of claim 35, wherein each of the different nucleotides of said combinations is either a different native nucleotide, or a different unlabeled nucleotide analog that is free of any exogenous fluorescent moiety.

37. The method of claim 21, wherein each different nucleotide of said combination is a different labeled nucleotide comprising an exogenous detectable label that produces a signal, and wherein the signal produced by each of the different labeled nucleotides is substantially the same before and after formation of the ternary complex.

38. The method of claim 21, wherein the plurality of polymerase-nucleotide combinations consists of four polymerase-nucleotide combinations.

39. The method of claim 21, wherein the primed template nucleic acid molecule is contained within a flow cell, and wherein step (b) comprises serially contacting the primed template nucleic acid molecule by flowing through the flow cell, one at a time, a plurality of reagent solutions, each comprising one of the plurality of polymerase-nucleotide combinations.

40. The method of claim 21, further comprising the step of removing the reversible terminator moiety from the 3' nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule comprising an extendable primer.

41. The method of claim 21, further comprising the step of (d) flowing through the flow cell a stripping buffer that dissociates the ternary complex to remove the polymerase and said one of the different nucleotides from the primed template nucleic acid molecule.

42. The method of claim 41, further comprising the step of (e) removing the reversible terminator moiety attached to the 3' terminal nucleotide of the primer of the primed template nucleic acid molecule to produce a primed template nucleic acid molecule comprising an extendable primer.

43. The method of claim 42, further comprising the step of (f) incorporating a nucleotide into the extendable primer.

44. The method of claim 43, wherein the nucleotide incorporated into the extendable primer comprises a reversible terminator moiety.

45. The method of claim 44, further comprising repeating steps (a)-(f) at least once to obtain sequence information for the template nucleic acid molecule.

46. A method of determining the identity of the next correct nucleotide for a primed template nucleic acid molecule, the method comprising:

(a) providing a template nucleic acid molecule primed with a primer;

(b) contacting the primed template nucleic acid molecule from step (a) with a first reaction mixture comprising a polymerase and at least one test nucleotide under conditions that,

(i) stabilize ternary complexes comprising the primed template nucleic acid molecule, the polymerase and a next correct nucleotide, while precluding incorporation of any nucleotide into the primer, and

(ii) destabilize binary complexes comprising the primed template nucleic acid molecule and the polymerase but not the next correct nucleotide;

(c) detecting interaction of the polymerase with the primed template nucleic acid molecule without chemical incorporation of any nucleotide into the primer of the primed template nucleic acid molecule, to determine whether a ternary complex formed in step (b); and

(d) determining whether any of the test nucleotides is the next correct nucleotide for the primed template nucleic acid molecule using the detected interaction of step (c).

47. The method of claim 46, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by including in the first reaction mixture a non-catalytic metal ion that inhibits polymerization.

48. The method of claim 46, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by including in the first reaction mixture a polymerase inhibitor selected from the group consisting of an allosteric

polymerase inhibitor, an uncompetitive polymerase inhibitor, a competitive polymerase inhibitor, and a non-competitive polymerase inhibitor.

49. The method of claim 46, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by terminating the primer with a reversible terminator nucleotide before conducting step (b).

50. The method of claim 46, wherein the conditions that destabilize binary complexes in step (b) comprise a concentration of from 50 mM to 1,500 mM of a salt that provides monovalent cations, said salt being included in the first reaction mixture.

51. The method of claim 50, wherein the reaction conditions that stabilize ternary complexes and destabilize binary complexes in step (b) enhance ternary complex formation over binary complex formation by at least two-fold.

52. The method of claim 51, wherein the reaction conditions that stabilize ternary complexes and destabilize binary complexes in step (b) enhance ternary complex formation over binary complex formation by at least five-fold.

53. The method of claim 50, wherein the salt that provides monovalent cations further provides glutamate anions.

54. The method of claim 50, wherein the concentration of the salt that provides monovalent cations is from 50 mM to 500 mM.

55. The method of claim 54, wherein the concentration of the salt that provides monovalent cations is from 100 mM to 300 mM.

56. The method of claim 54, wherein the first reaction mixture further comprises a glutamate salt at a concentration of from 10 mM to 1.6 M.

57. The method of claim 56, wherein the concentration of the glutamate salt is from 80 mM to 320 mM.

58. The method of claim 54, wherein the salt is a glutamate salt.

59. The method of claim 54, wherein the salt is selected from the group consisting of NaCl, KCl, $NH_2(SO_4)$, and potassium glutamate.

60. The method of claim 50, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by a non-catalytic metal ion that inhibits polymerization.

61. The method of claim 50, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by terminating the primer with a reversible terminator nucleotide before performing step (b).

62. The method of claim 54, wherein the conditions that destabilize binary complexes in step (b) comprise 200 mM of the salt that provides monovalent cations.

63. The method of claim 62, wherein the salt is selected from the group consisting of NaCl, KCl, $NH_2(SO_4)$, and potassium glutamate.

64. The method of claim 62, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by a non-catalytic metal ion that inhibits polymerization.

65. The method of claim 64, wherein the first reaction mixture comprises potassium glutamate at a concentration between 10 mM and 1.6 M.

66. The method of claim 62, wherein the conditions that stabilize ternary complexes while precluding incorporation of any nucleotide into the primer are provided by terminating the primer with a reversible terminator nucleotide before performing step (b).

67. The method of claim 46, wherein each of the test nucleotides of the first reaction mixture is a different unlabeled nucleotide.

68. The method of claim 67, wherein each of the different unlabeled nucleotides is a different native nucleotide.

69. The method of claim 67, wherein the polymerase comprises an exogenous detectable label.

70. The method of claim 69, wherein the exogenous detectable label comprises a fluorescent label.

71. The method of claim 67,

wherein the polymerase comprises a detectable label that emits a signal,

wherein emission of the signal by the detectable label is substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide.

72. The method of claim 46,

wherein the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support,

wherein the polymerase comprises a detectable label that emits a signal,

wherein emission of the signal by the detectable label is substantially uniform when the polymerase is complexed with the primed template nucleic acid molecule in the presence or absence of any nucleotide,

wherein step (c) comprises measuring intensity of the signal at the locus on the solid support, and

wherein increased formation of the ternary complex in step (b) is indicated by increased intensity of the signal at the locus.

73. The method of claim 72, wherein the solid support is contained within a flow cell, and wherein the detectable label is a fluorescent detectable label.

74. The method of claim 72, further comprising the step of:

(e) replacing the first reaction mixture with a second reaction mixture comprising a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of said one or more nucleotides incorporates into the primer.

75. The method of claim 74, wherein the one or more nucleotides of the second reaction mixture comprises at least one reversible terminator nucleotide.

76. The method of claim 75, wherein the solid support is contained within a flow cell, and wherein step (e) comprises replacing the first reaction mixture by fluid flow through the flow cell.

77. The method of claim 74, wherein the second reaction mixture comprises less than 100 mM of each of NaCl and KCl.

78. The method of claim 74, wherein the polymerase of the second reaction mixture is a different polymerase than the polymerase of the first reaction mixture.

79. The method of claim 75, wherein the reversible terminator nucleotide does not comprise a fluorescent label.

80. The method of claim 46,

wherein the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support contained within a flow cell, and

wherein the method further comprises:

(e) replacing, by fluid flow through the flow cell, the first reaction mixture with a second reaction mixture comprising a polymerase, one or more nucleotides, and a catalytic cation, whereby at least one of said one or more nucleotides incorporates into the primer.

81. The method of claim 80, wherein the one or more nucleotides of the second reaction mixture comprises at least one reversible terminator nucleotide.

82. The method of claim 80, wherein the second reaction mixture comprises less than 100 mM of each of NaCl and KCl.

83. The method of claim 80, further comprising a step for washing the immobilized primed template nucleic acid molecule between step (c) and step (e) to remove at least one of the components of the first reaction mixture.

84. The method of claim 80, wherein the polymerase of the second reaction mixture is a different polymerase than the polymerase of the first reaction mixture.

85. The method of claim 81, wherein the reversible terminator nucleotide does not comprise a fluorescent label.

86. The method of any of claims 46,

wherein the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support,

wherein step (c) comprises measuring intensity of a signal indicating interaction of the polymerase with the primed template nucleic acid molecule at the locus on the solid support, and

wherein step (d) comprises determining that one of the test nucleotides of the first reaction mixture is the next correct nucleotide for the primed template nucleic acid molecule when the measured intensity of the signal exceeds a predetermined threshold.

87. The method of claim 86, further comprising the step of:

washing the immobilized primed template nucleic acid molecule after step (c) and before step (e) to remove at least one of the components of the first reaction mixture.

88. The method of claim 46, wherein the primed template nucleic acid molecule of step (a) is immobilized at a locus on a solid support contained within a flow cell, and wherein the method further comprises the steps of:

washing the immobilized primed template nucleic acid molecule after step (c) to remove one or more of said at least one test nucleotide of the first reaction mixture; and

detecting interaction of the polymerase with the immobilized primed template nucleic acid molecule after the washing step to determine whether there remains any of the ternary complex that may have formed in step (b).

89. A method of delivering polymerase and nucleotide to a population of immobilized nucleic acid features, each feature comprising a primed template nucleic acid molecule, the method comprising the steps of:

(a) contacting the population of immobilized nucleic acid features with a first reagent comprising a polymerase, a first nucleotide, and at least one non-immobilized primed template nucleic acid molecule,

wherein the first nucleotide is not the next correct nucleotide for any of said non-immobilized primed template nucleic acid molecules of the first reagent,

wherein the contacting takes place under conditions that stabilize ternary complexes and inhibit or preclude catalysis of phosphodiester bond formation by the polymerase, and

whereby, compared to the use of reagents comprising the polymerase and the first nucleotide in the absence of said non-immobilized primed template nucleic acid molecule of the first reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule is reduced.

90. The method of claim 89, wherein after step (a) there is the further step of

(b) replacing the first reagent with a second reagent comprising the polymerase of the first reagent, a second nucleotide, and at least one non-immobilized primed template nucleic acid molecule,

wherein the first and second nucleotides are different from each other,

wherein the second nucleotide is not the cognate nucleotide for any of said non-immobilized primed template nucleic acid molecules of the second reagent,

wherein the replacing takes place under conditions that stabilize ternary complexes and inhibit or preclude catalysis of phosphodiester bond formation by the polymerase, and

whereby, compared to the use of reagents comprising the polymerase and the second nucleotide in the absence of said non-immobilized primed template nucleic acid molecule of the second reagent, nucleotide-independent polymerase binding to the primed template nucleic acid molecule is reduced.

91. The method of claim 90, wherein the non-immobilized primed template nucleic acid molecules of the first and second reagents are different from each other.

92. A reaction mixture, comprising:

(a) a plurality of immobilized nucleic acid features, each feature comprising an immobilized primed template nucleic acid;

(b) a polymerase;

(c) a non-immobilized primed template nucleic acid molecule; and

(d) a nucleotide,

wherein the nucleotide is not the cognate nucleotide for the non-immobilized primed template nucleic acid molecule, and

wherein nucleotide-independent binding of the polymerase to the immobilized primed template nucleic acid is reduced by the presence of the non-immobilized primed template nucleic acid molecule.

93. The reaction mixture of claim 92, wherein the polymerase, the immobilized primed template nucleic acid, and the nucleotide form a stabilized ternary complex that is inhibited or precluded from incorporating the nucleotide into the immobilized primed template nucleic acid.

94. A polymerase delivery reagent, comprising:

(a) a polymerase;

(b) at least one non-immobilized primed template nucleic acid molecule; and

(c) at least one nucleotide,

wherein none of said nucleotides is a next correct nucleotide for any of said non-immobilized primed template nucleic acid molecules.

95. The polymerase delivery reagent of claim 94, further comprising (d) a ternary complex stabilizing agent that inhibits phosphodiester bond formation by the solution-phase polymerase.

96. The polymerase delivery reagent of claim 94, wherein said at least one nucleotide comprises at least one native nucleotide.

97. The polymerase delivery reagent of claim 94, wherein the solution-phase primed template nucleic acid molecule comprises no more than three different types of primed template nucleic acid molecule, each with a different 3' terminal nucleotide.

98. The polymerase delivery reagent of claim 97, wherein the polymerase is a mutant polymerase without catalytic phosphodiester bond forming activity in the presence of $Mg^{2+}$ ions.

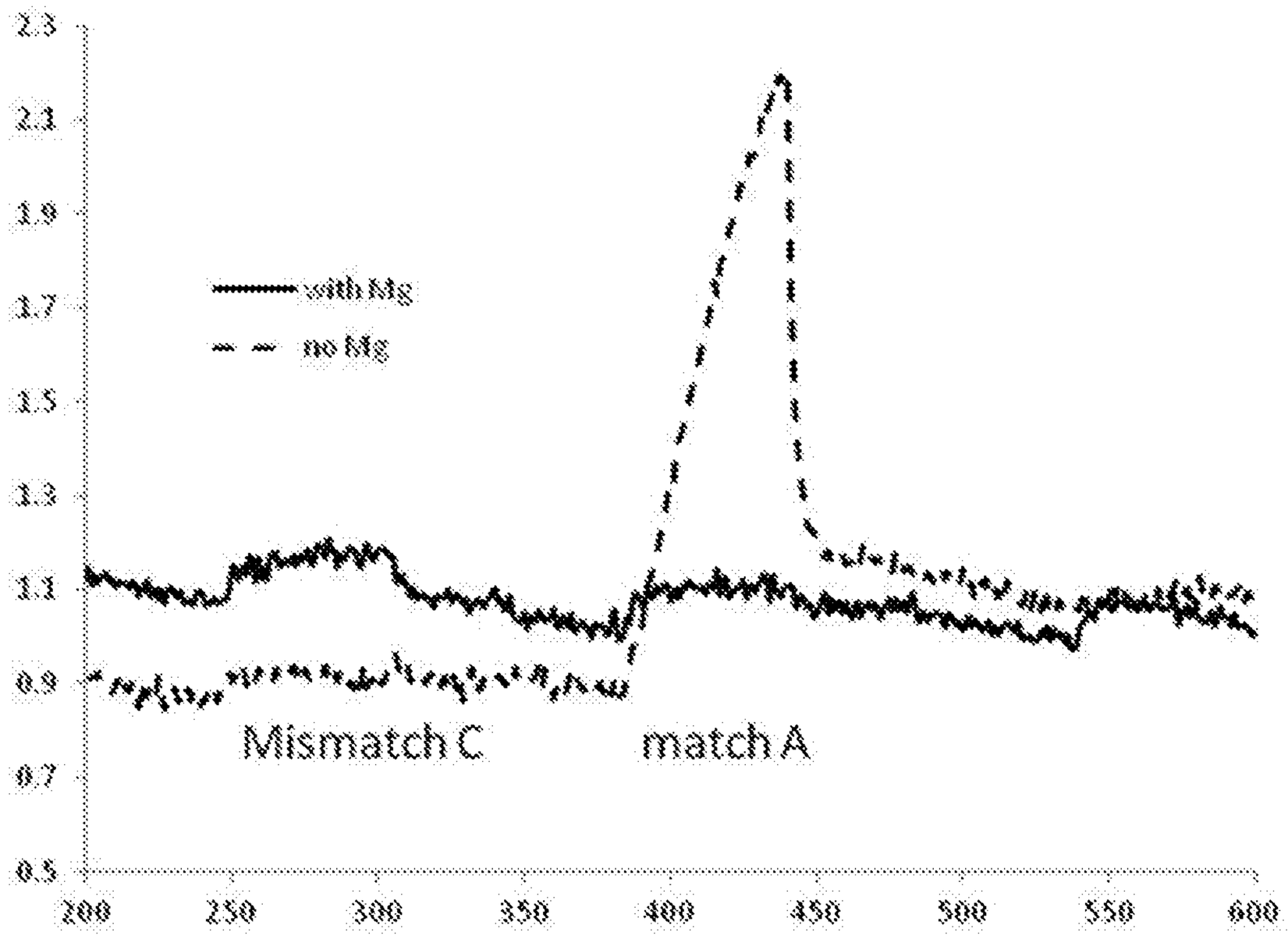

2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
200
250
300
350
400
450
500
550
600
with Mg
no Mg
Mismatch C
match A

FIG 1

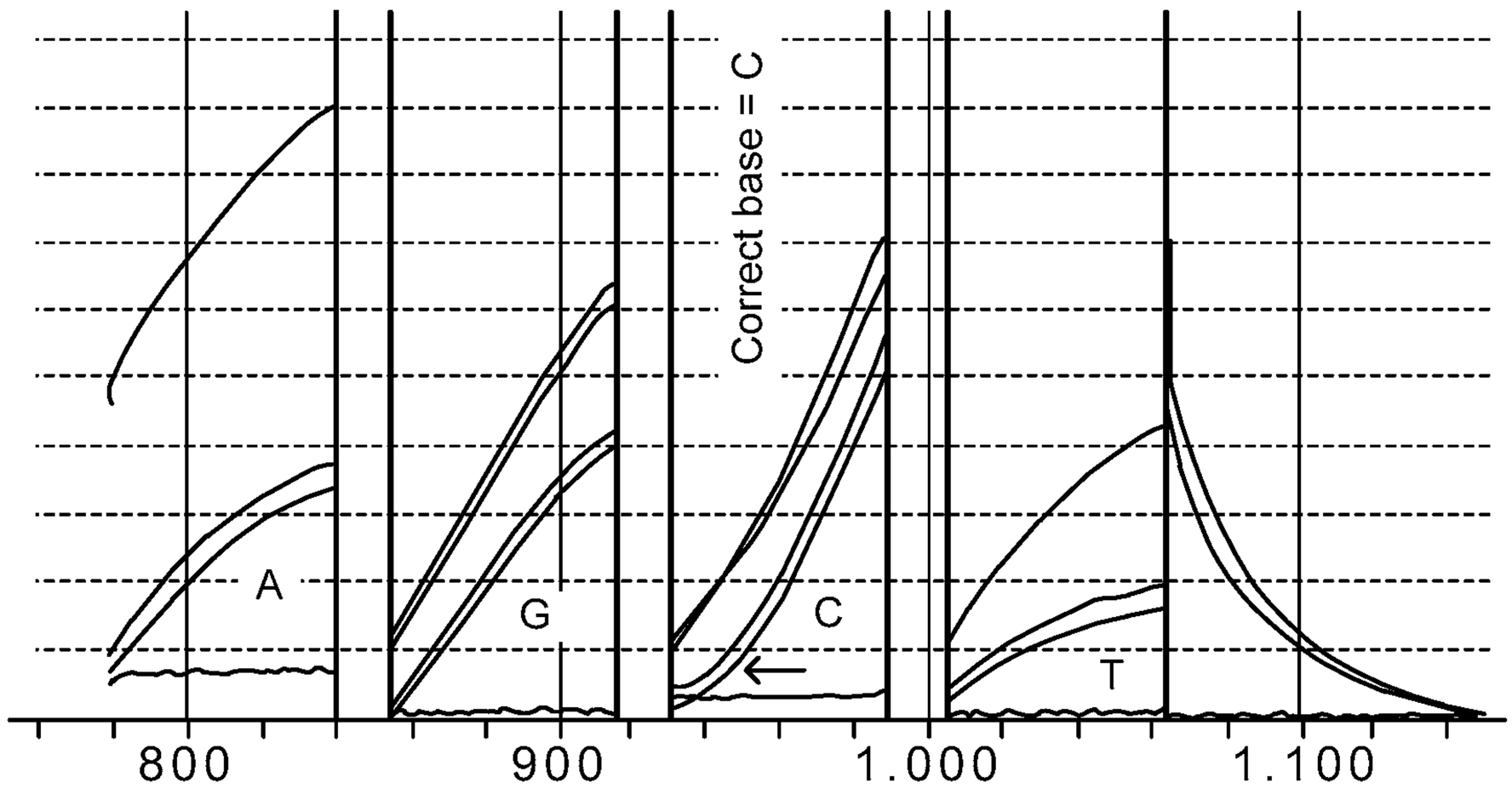

Correct base = C
A
G
C
T
800
900
1.000
1.100

FIG. 2

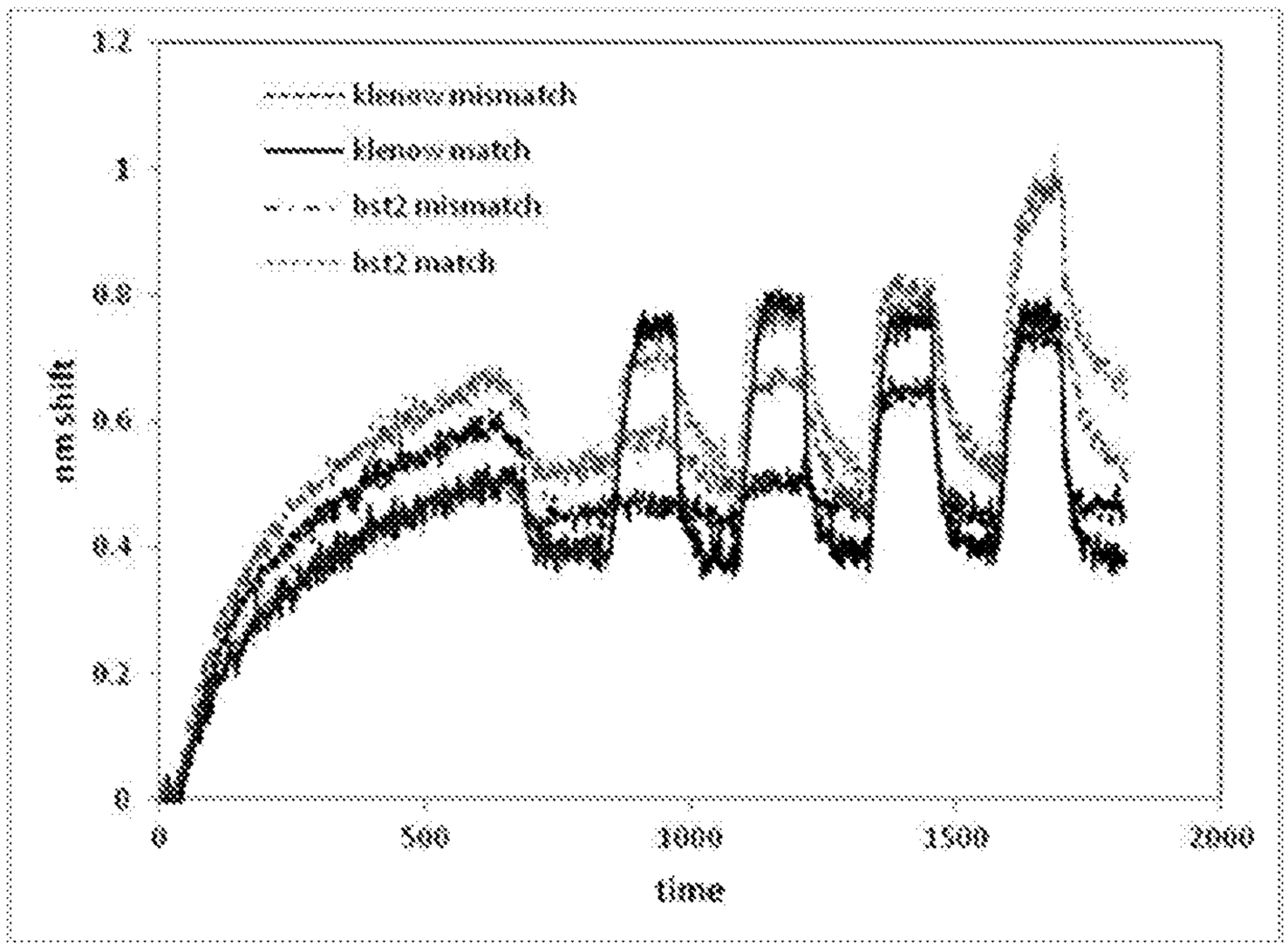

1.2
1
0.8
0.6
0.4
0.2
0
nm shift
0
500
1000
1500
2000
time
blue mismatch
blue match
bst2 mismatch
bst2 match

FIG. 3

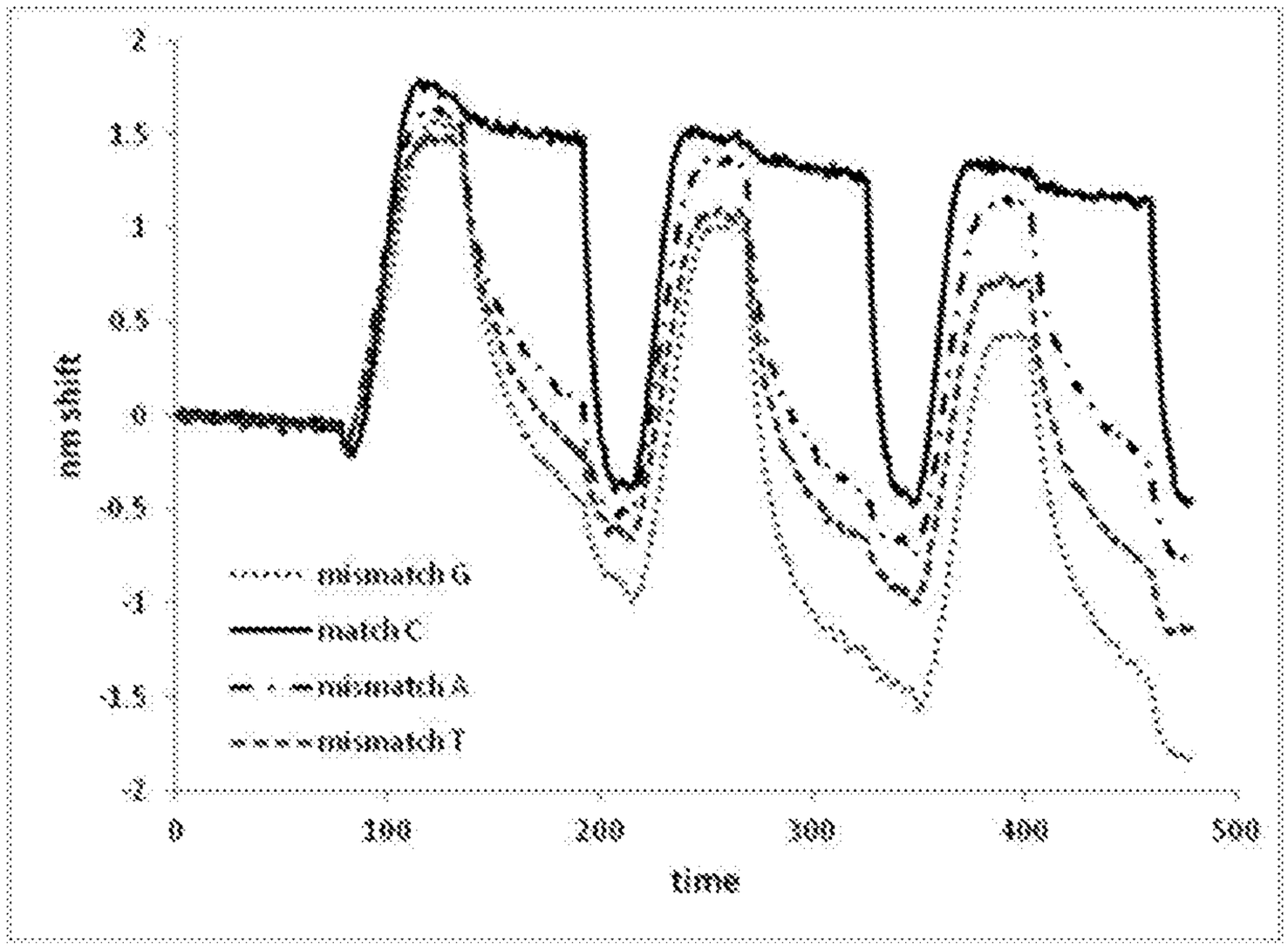

2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
nm shift
0
100
200
300
400
500
time
mismatch G
match C
mismatch A
mismatch T

FIG. 4

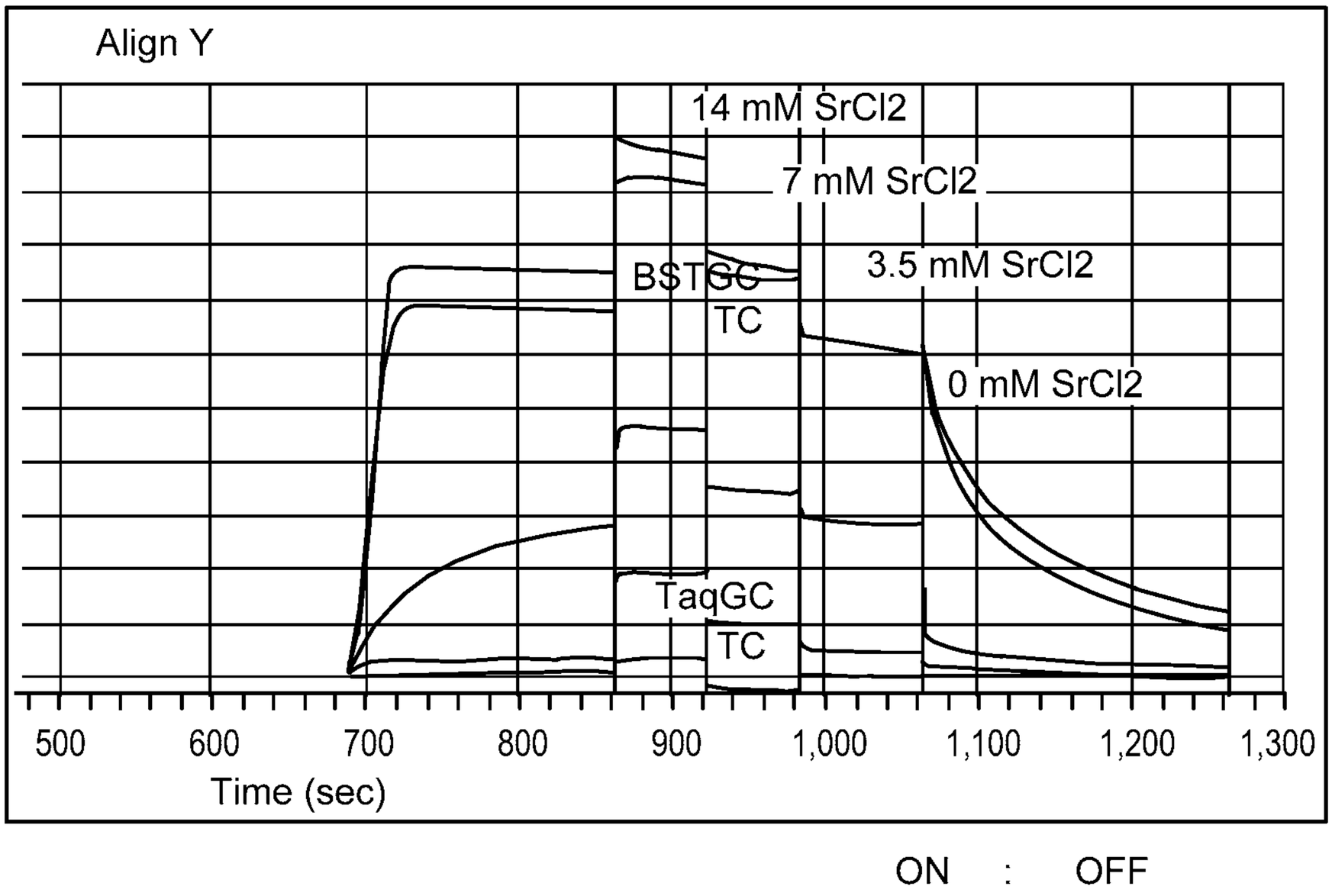

Align Y
14 mM SrCl2
7 mM SrCl2
3.5 mM SrCl2
BSTGC
TC
0 mM SrCl2
TaqGC
TC
500
600
700
800
900
1,000
1,100
1,200
1,300
Time (sec)
ON : OFF

**FIG. 5**

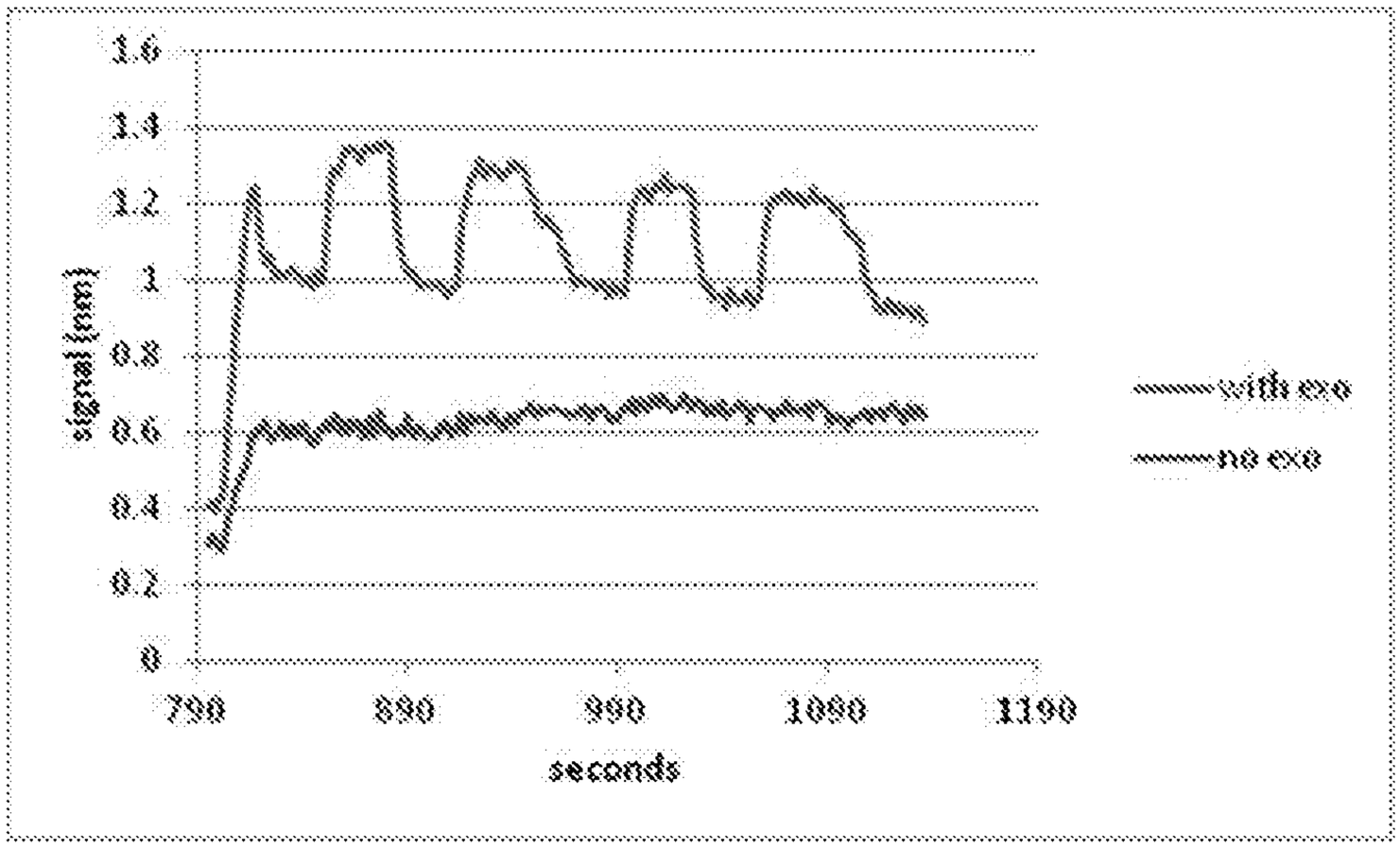

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
signal (mm)
790
890
990
1090
1190
seconds
with exo
no exo

**FIG. 6**

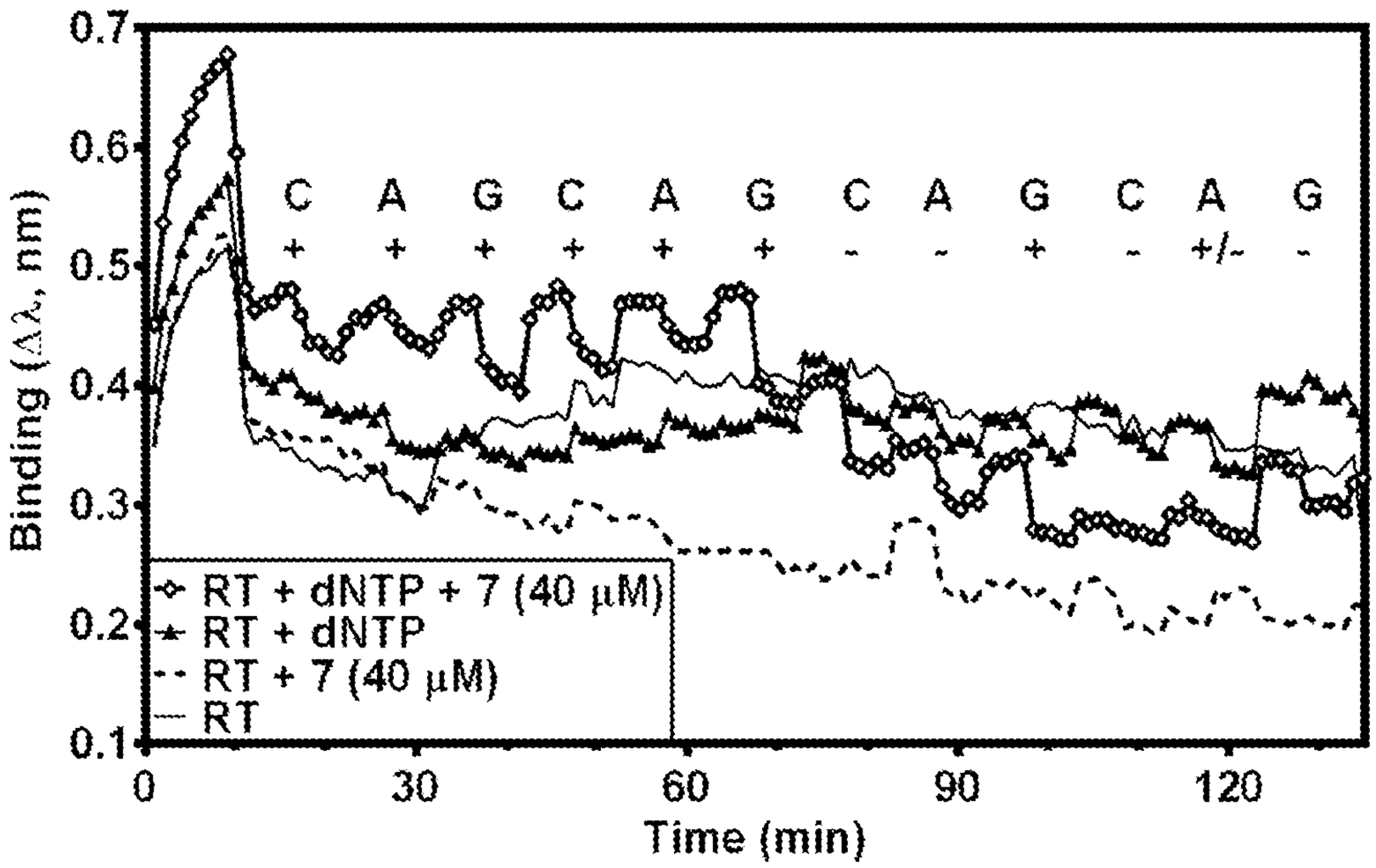

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Binding (Δλ, nm)
0
30
60
90
120
Time (min)
C A G C A G C A G C A G
+ + + + + + - - + - +/- -
RT + dNTP + 7 (40 μM)
RT + dNTP
RT + 7 (40 μM)
RT

FIG. 7A

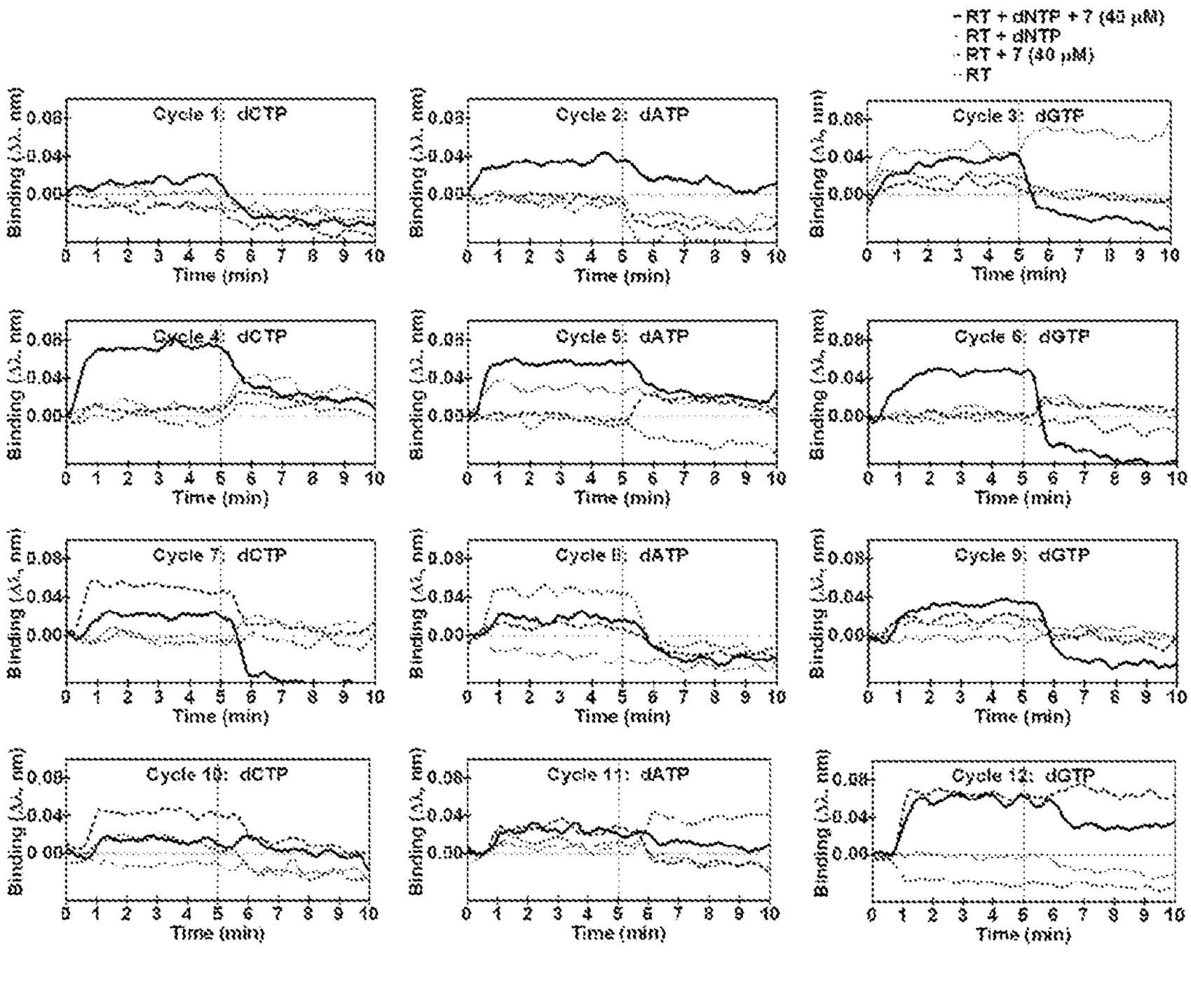

RT + dNTP + 7 (45 μM)
RT + dNTP
RT + 7 (30 μM)
RT
Cycle 1: dCTP
Cycle 2: dATP
Cycle 3: dGTP
Cycle 4: dCTP
Cycle 5: dATP
Cycle 6: dGTP
Cycle 7: dCTP
Cycle 8: dATP
Cycle 9: dGTP
Cycle 10: dCTP
Cycle 11: dATP
Cycle 12: dGTP
Binding (Δλ, nm)
Time (min)

**FIG. 7B**

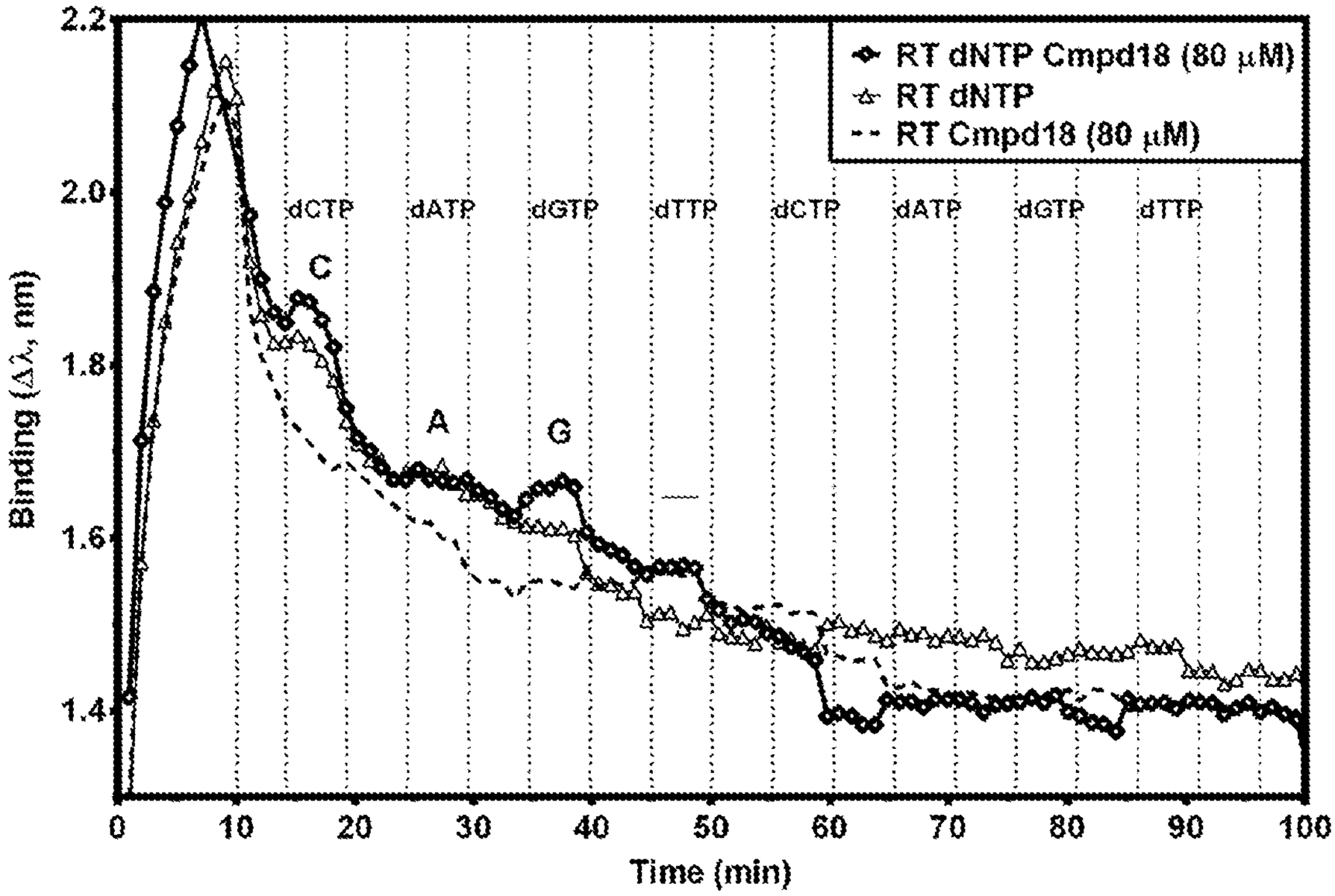

RT dNTP Cmpd18 (80 μM)
RT dNTP
RT Cmpd18 (80 μM)
dCTP
dATP
dGTP
dTTP
dCTP
dATP
dGTP
dTTP
C
A
G
T
Binding (Δλ, nm)
2.2
2.0
1.8
1.6
1.4
0
10
20
30
40
50
60
70
80
90
100
Time (min)

FIG. 8

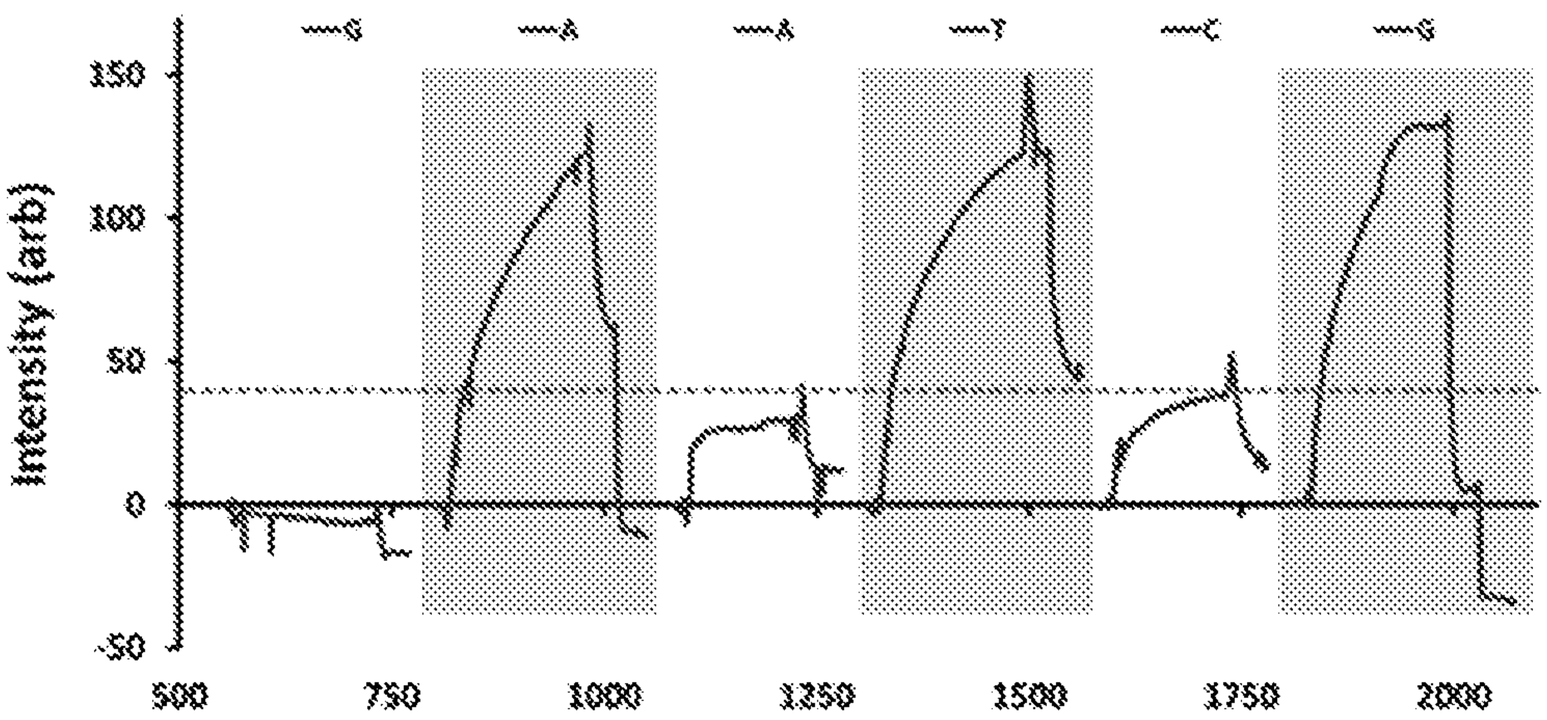

G
A
A
T
C
G
Intensity (arb)
150
100
50
0
-50
500
750
1000
1250
1500
1750
2000
Time (s)

FIG. 9

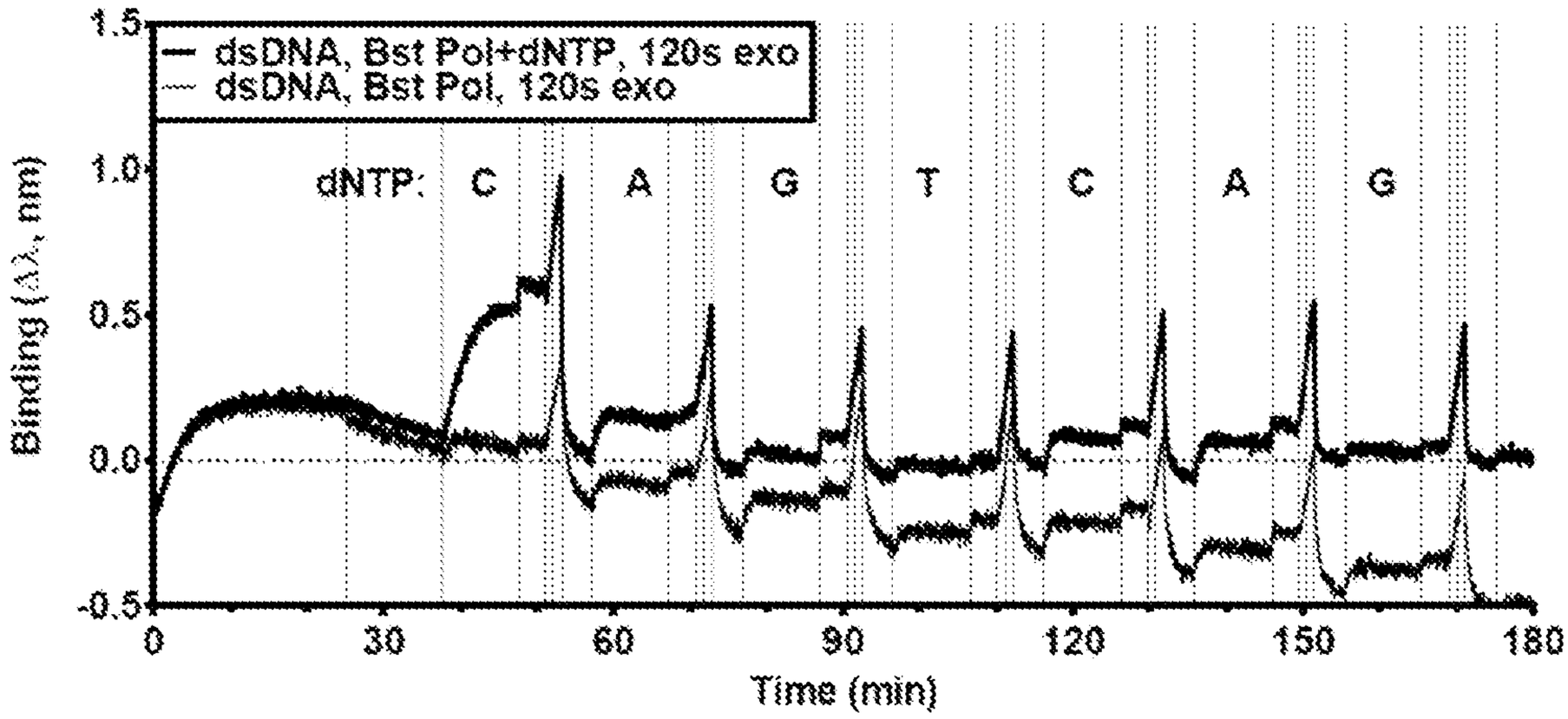



FIG. 10A

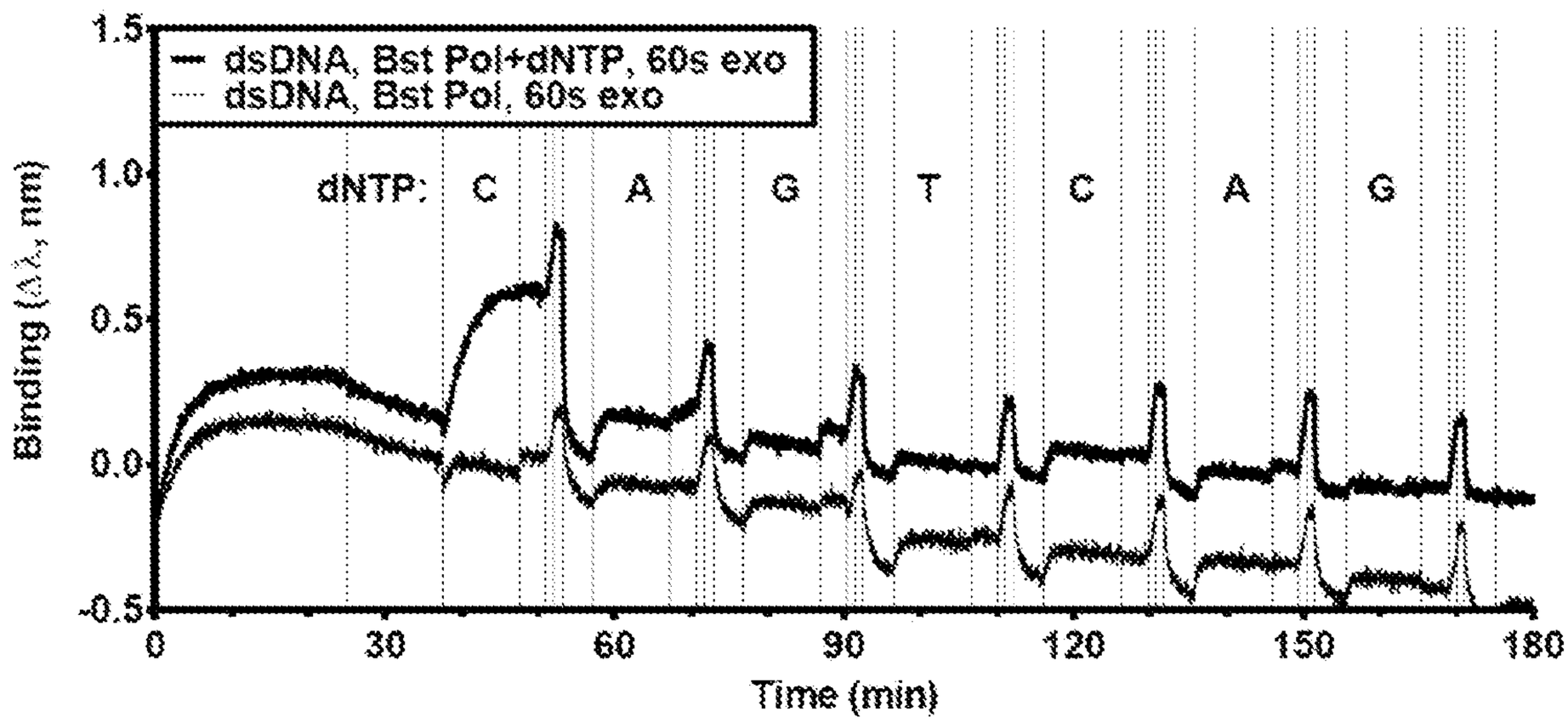



FIG. 10B

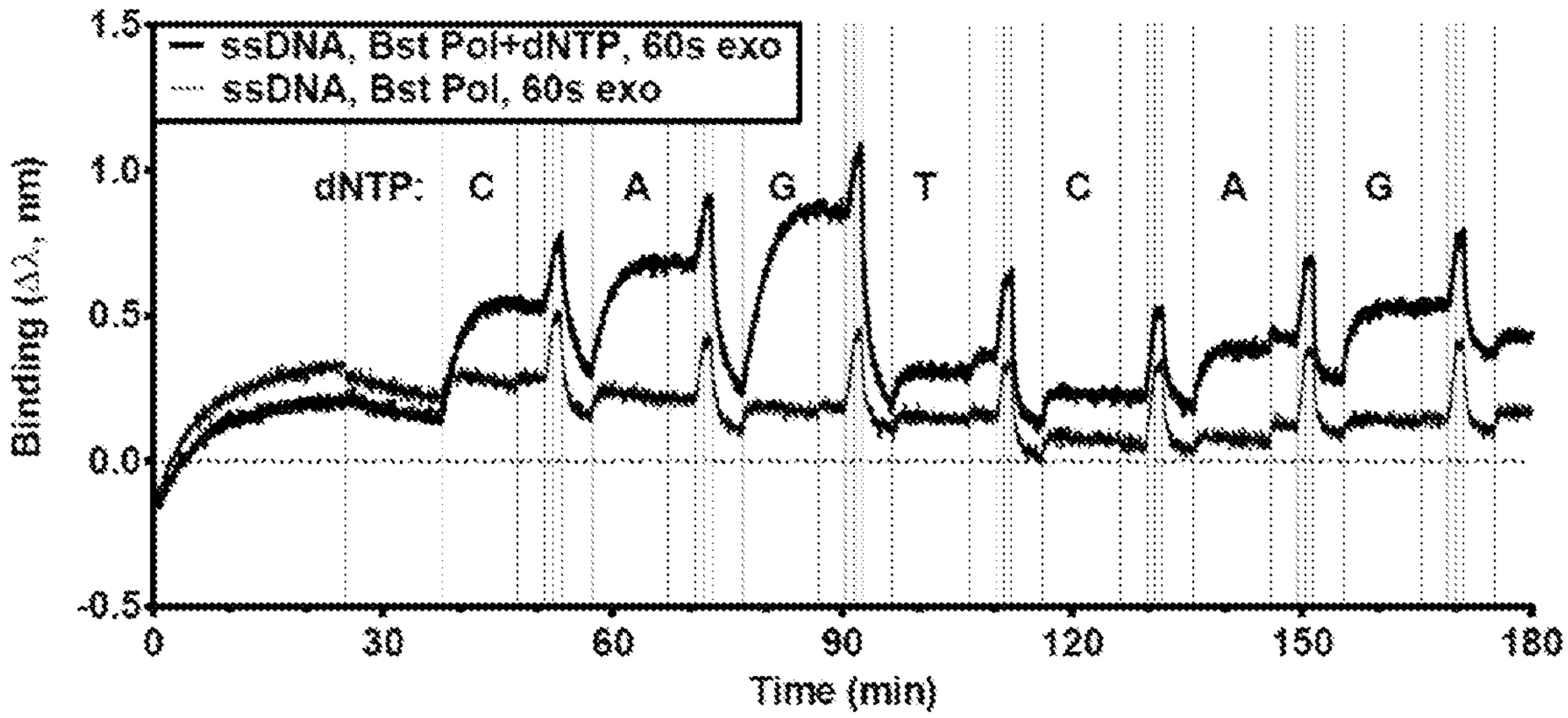



FIG. 10C

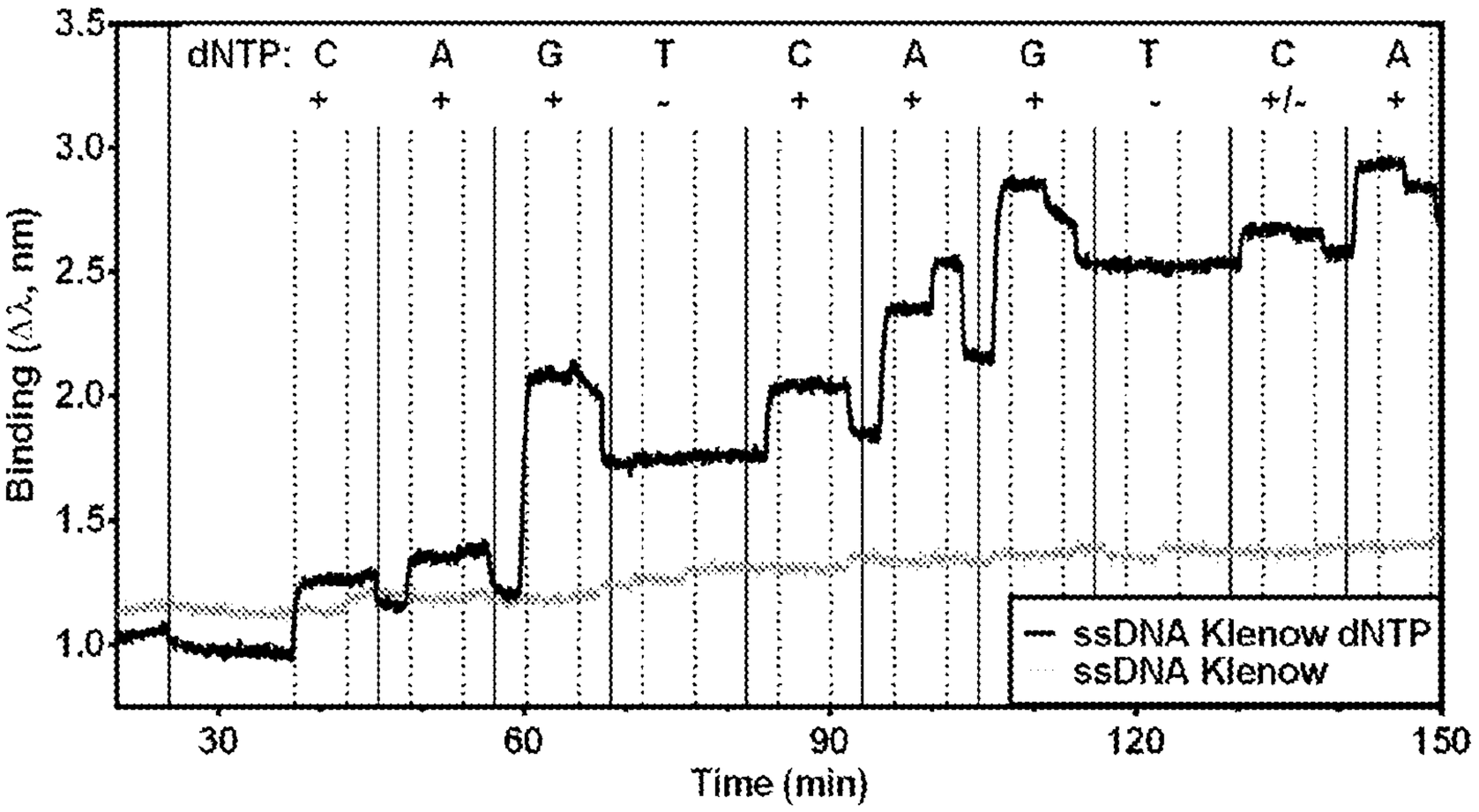



FIG. 11A

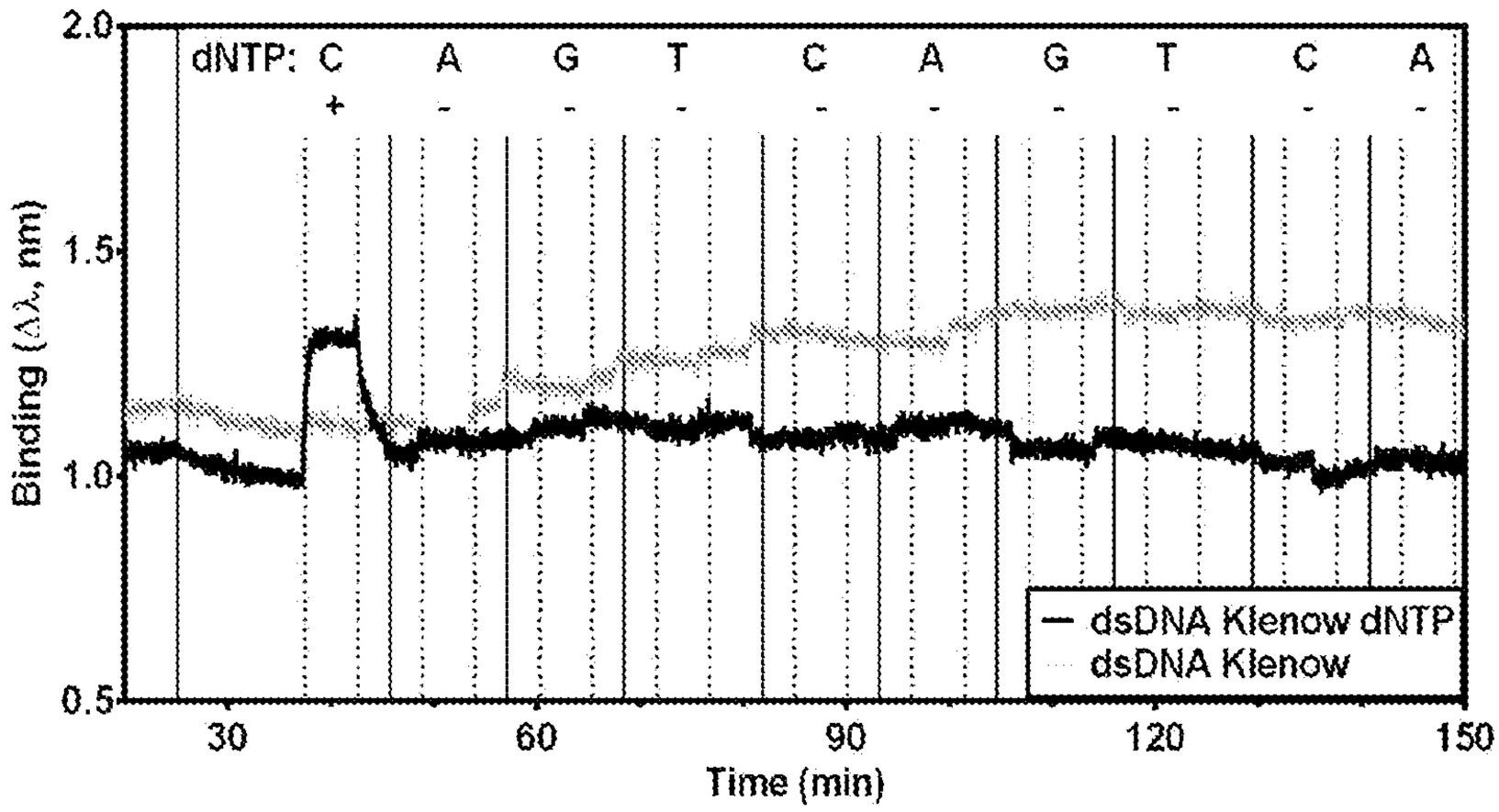



FIG. 11B

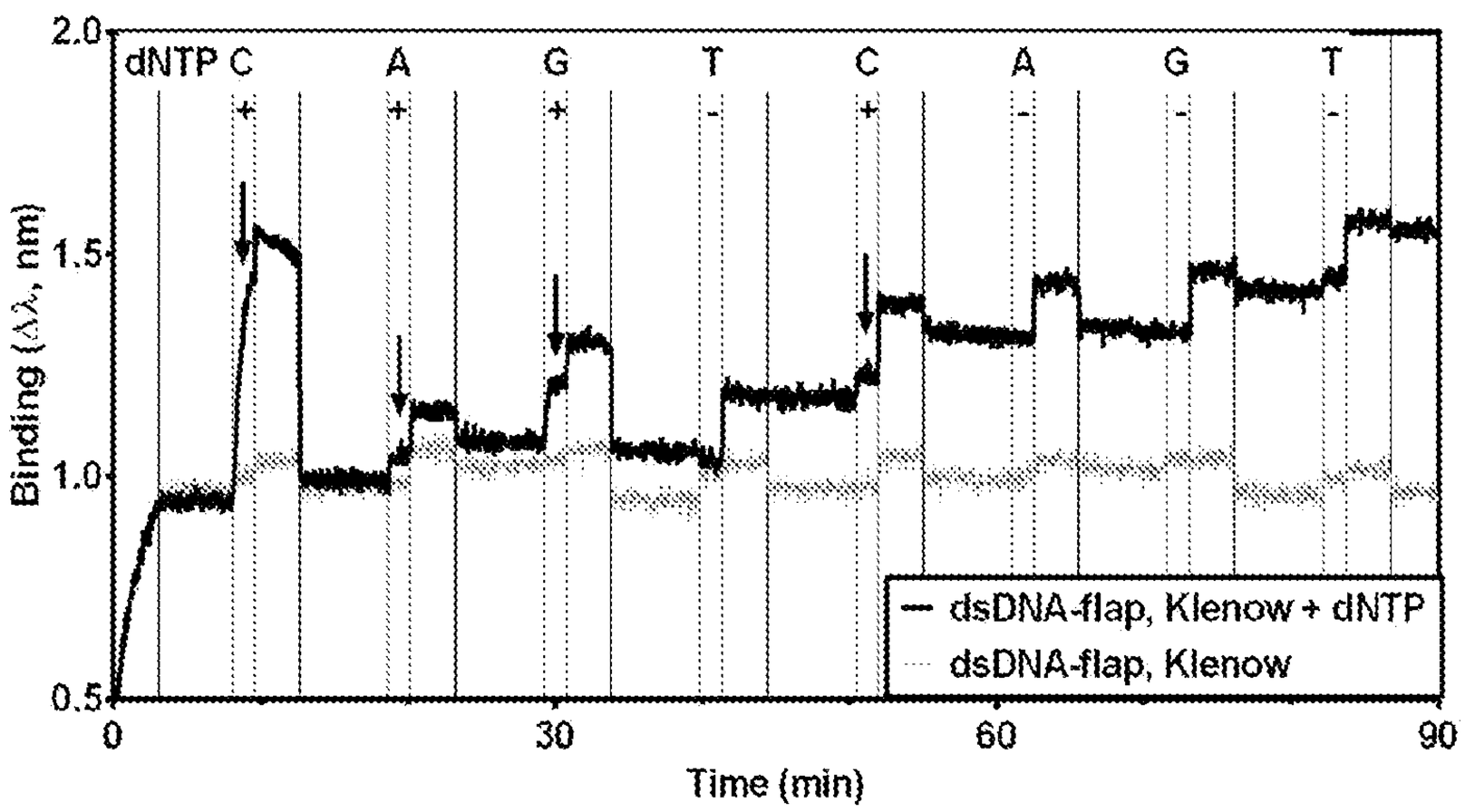



FIG. 11C

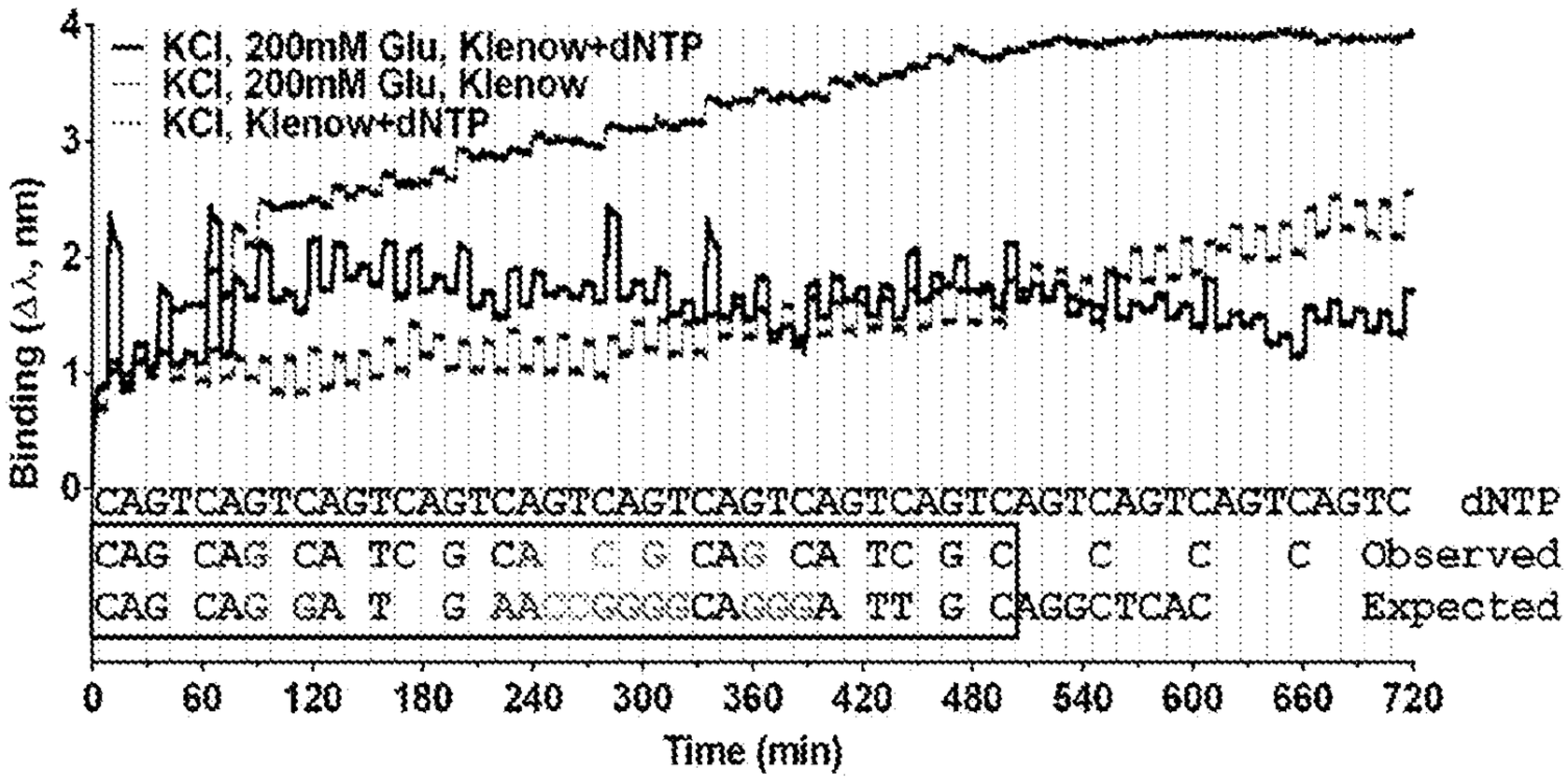



FIG. 12A

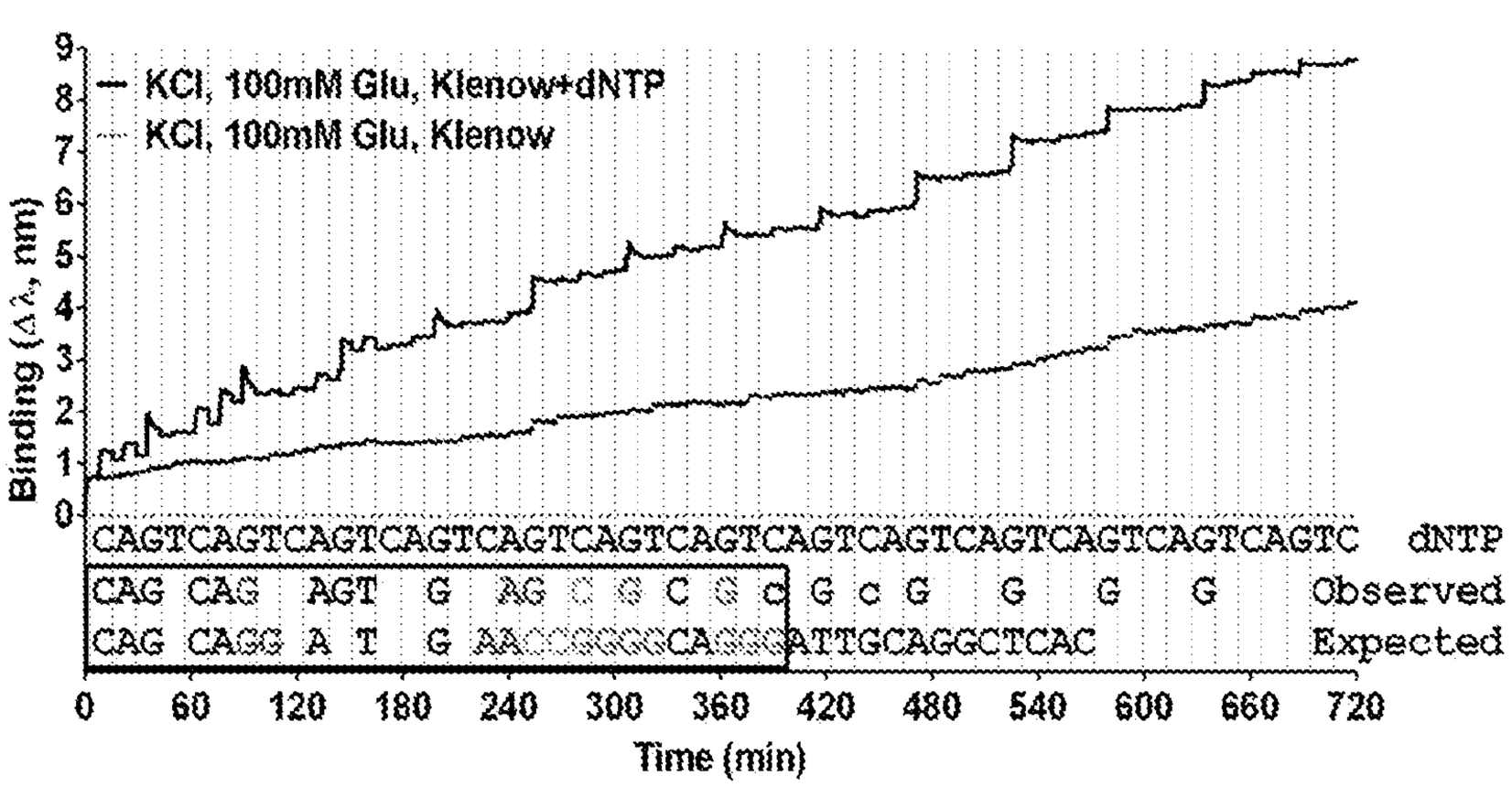



FIG. 12B

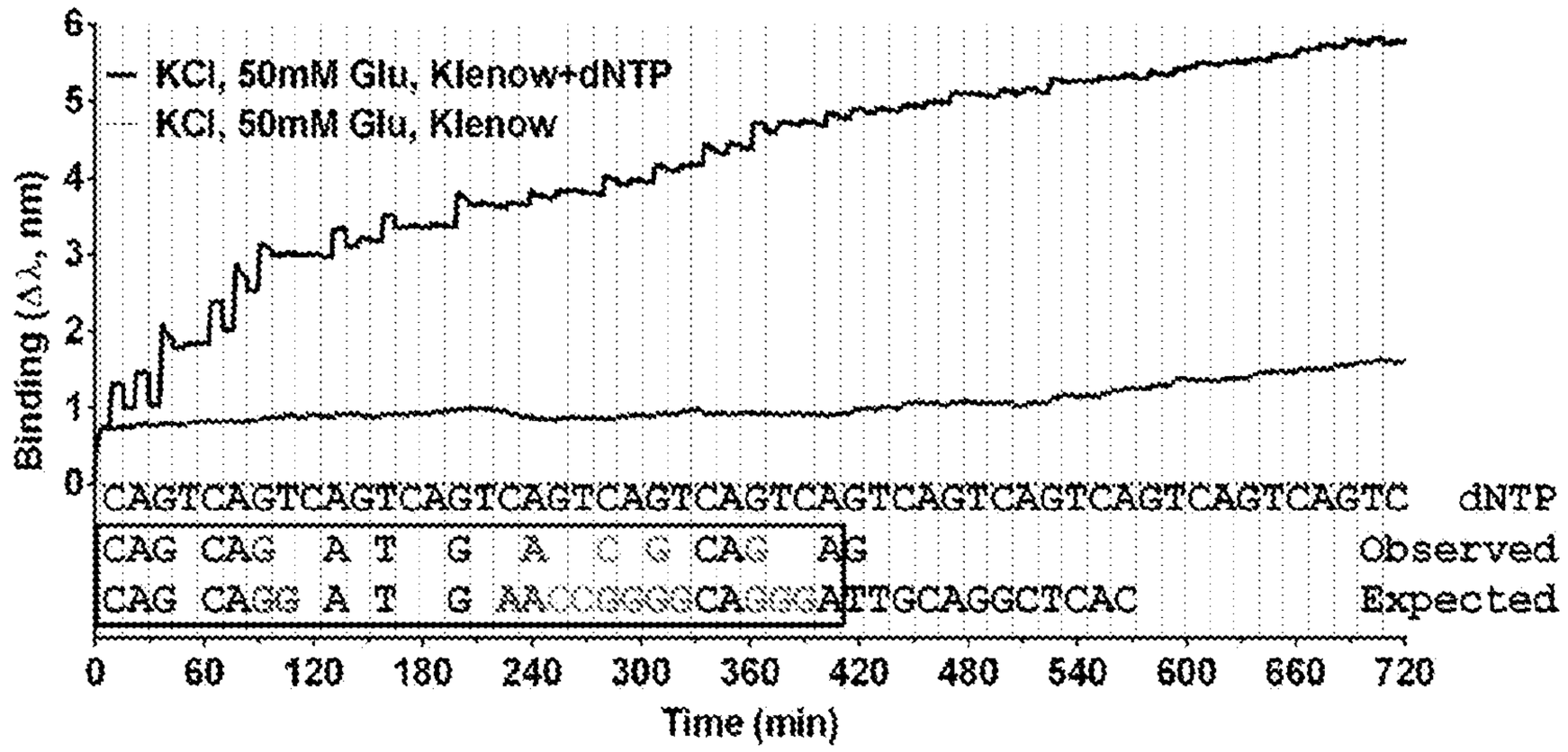



FIG. 12C

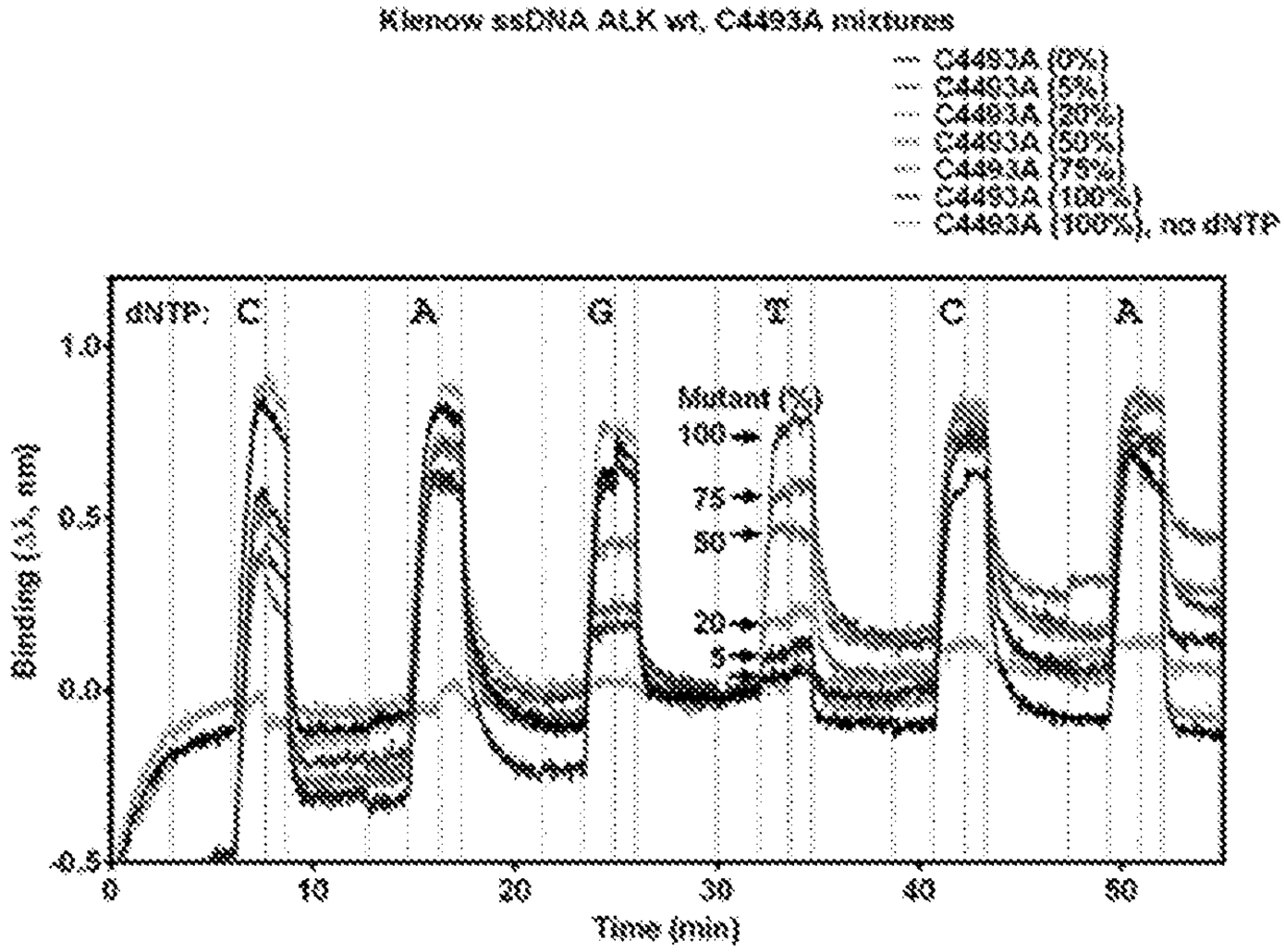



FIG. 13A

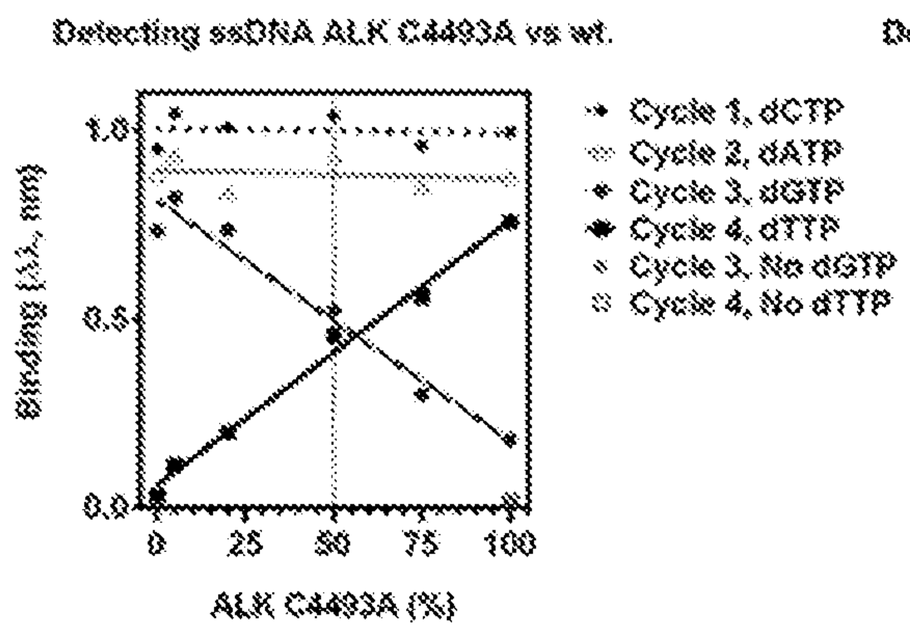



FIG. 13B

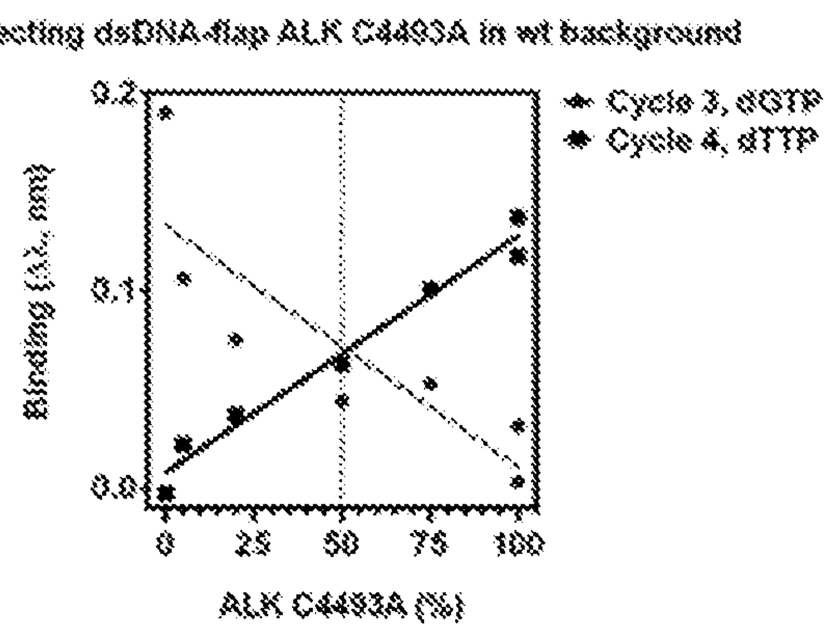



FIG. 13C

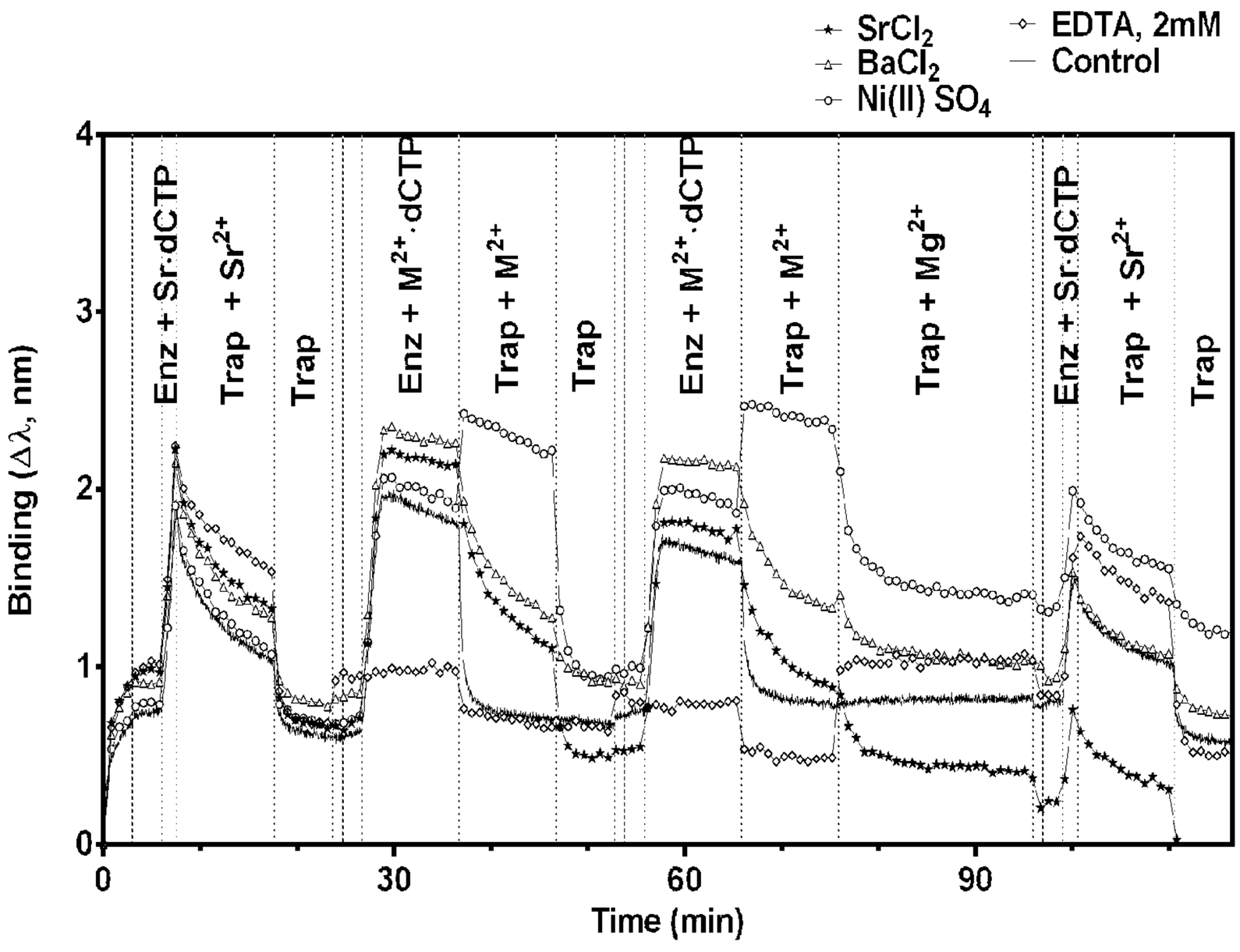



FIG. 14A

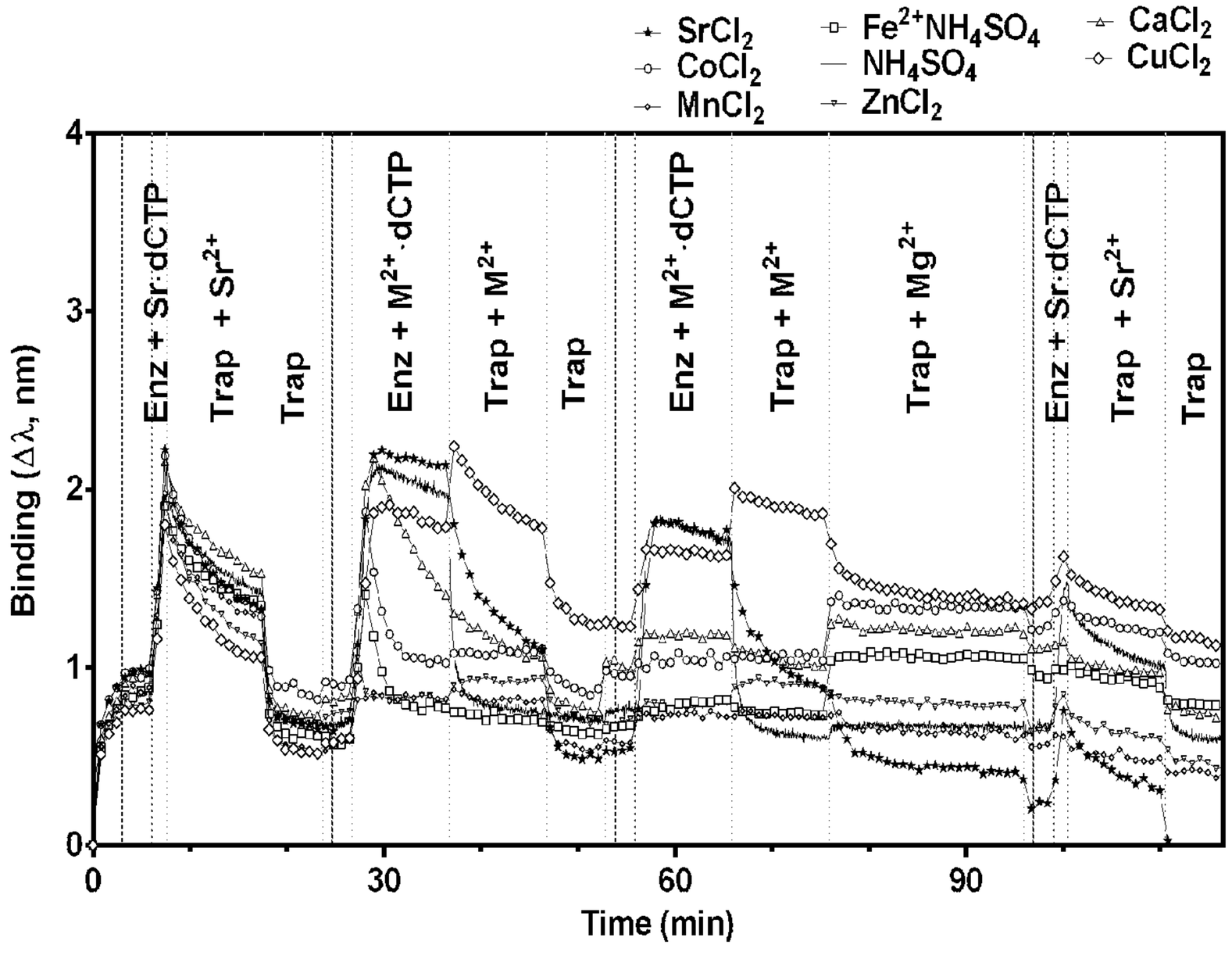
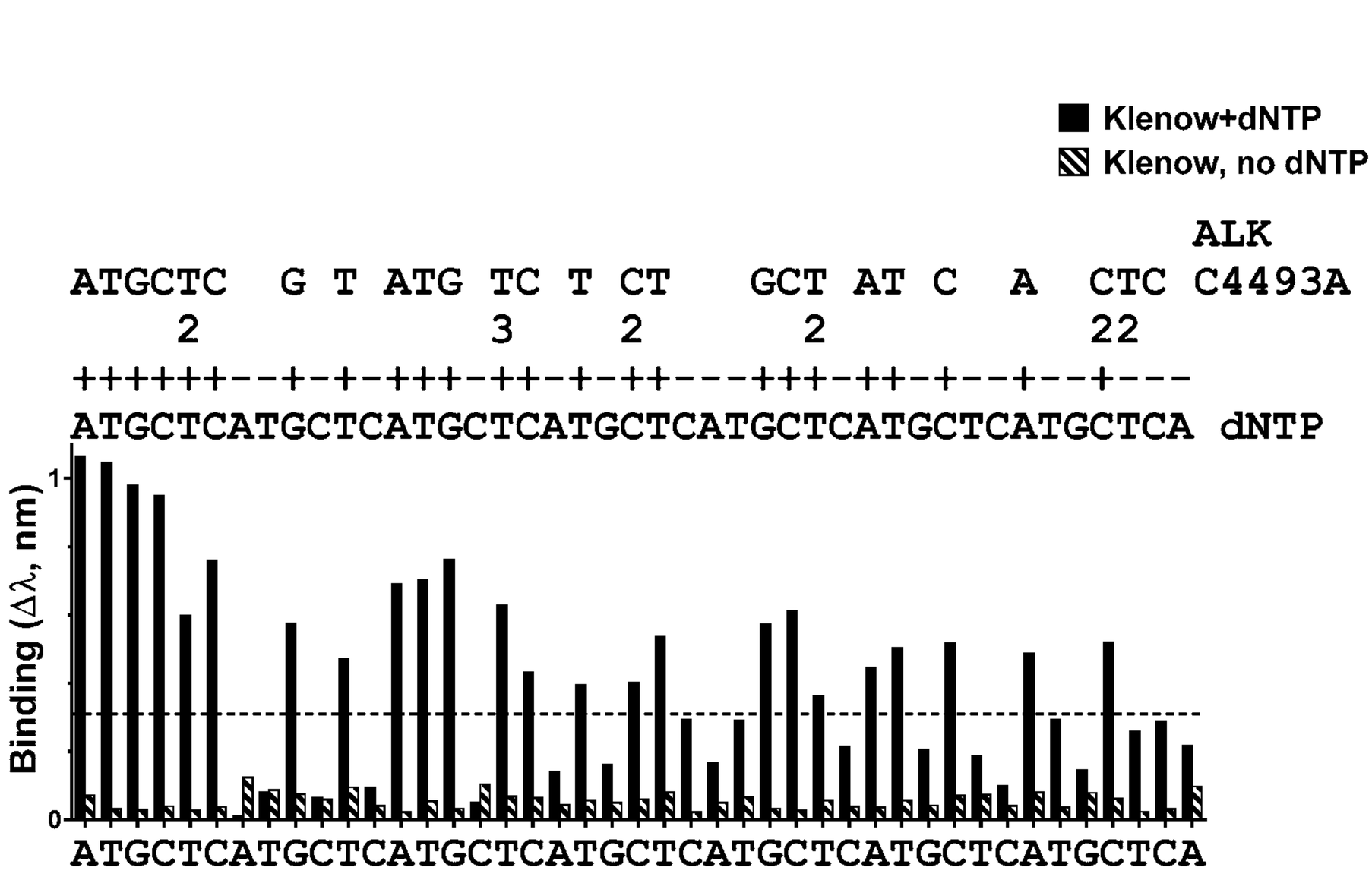



FIG. 14B




Double dNTP cycling

FIG. 15

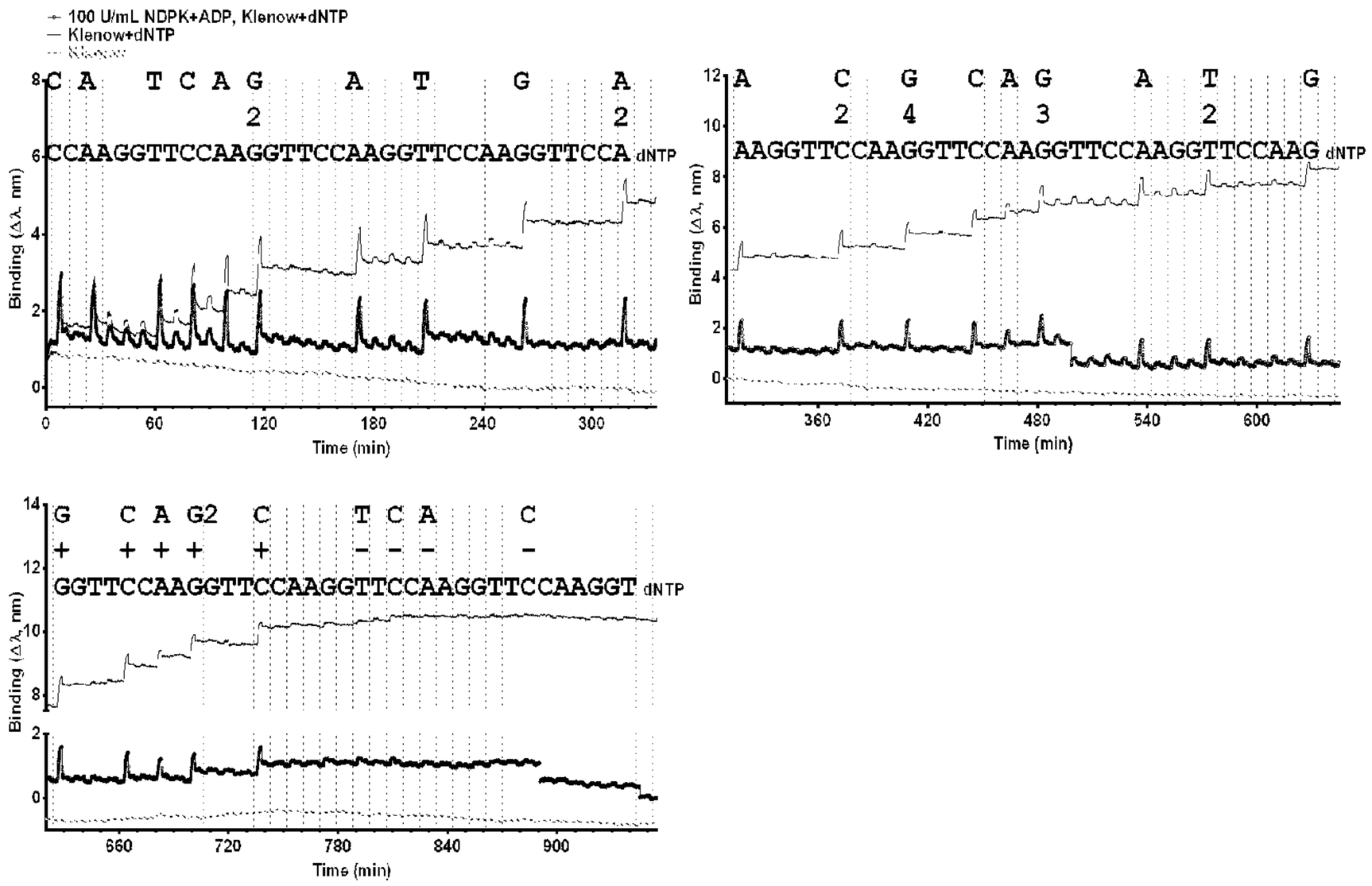



FIG. 16

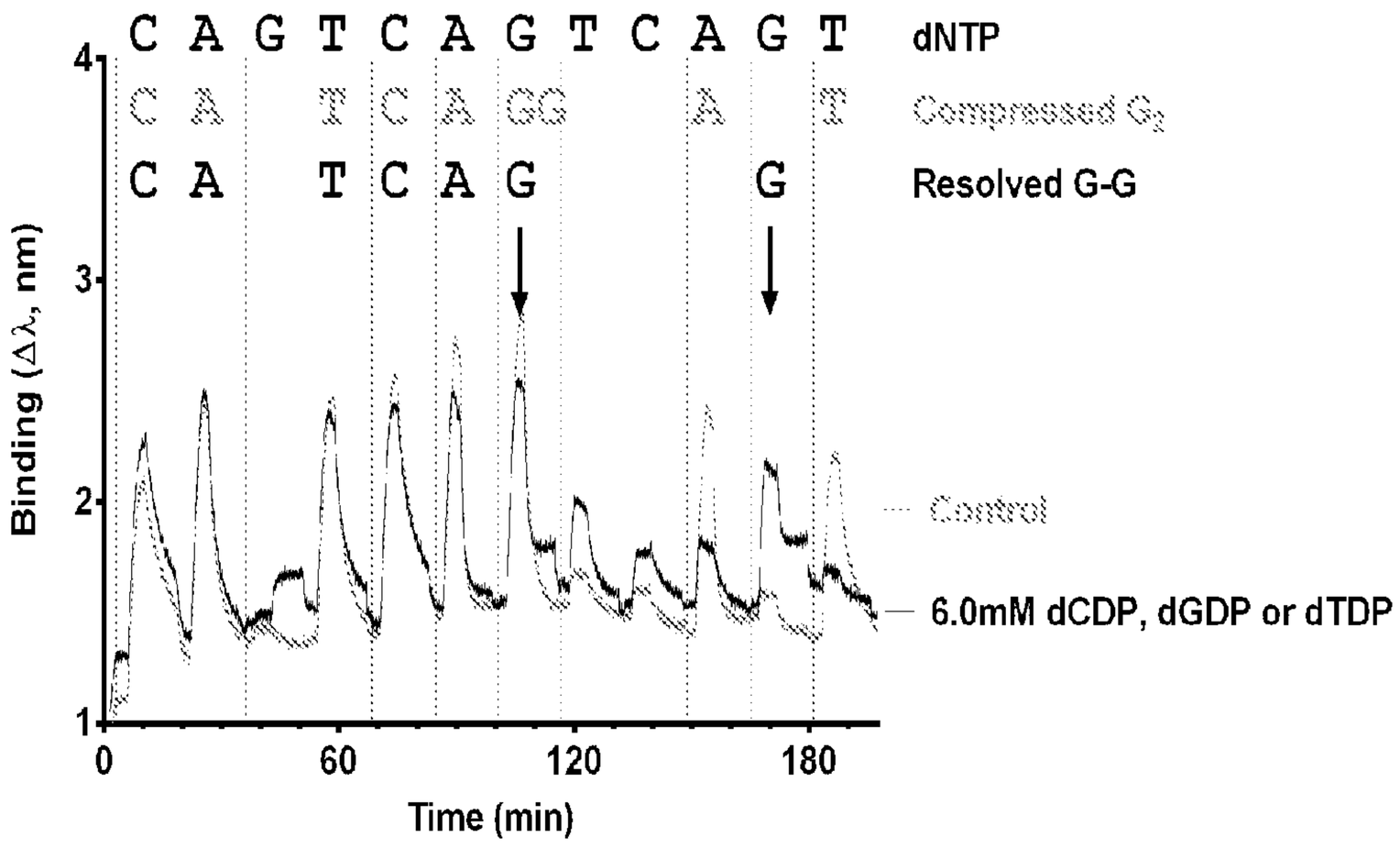



FIG. 17A

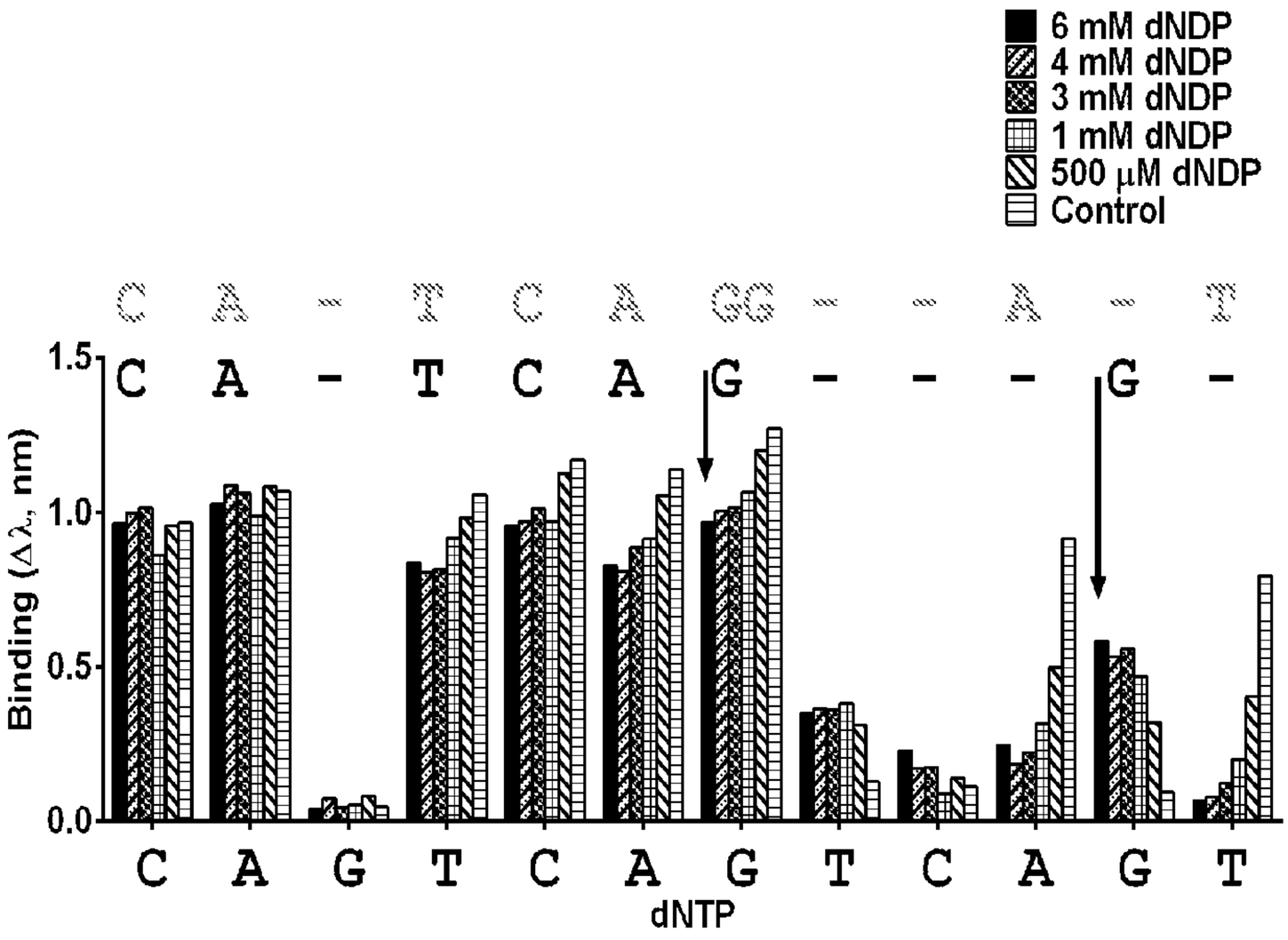



FIG. 17B

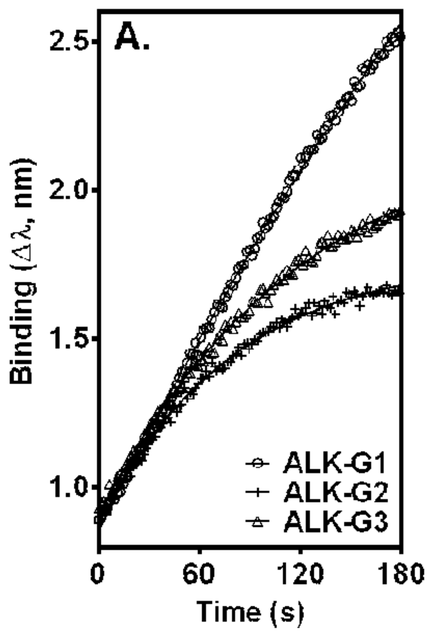



FIG. 18A

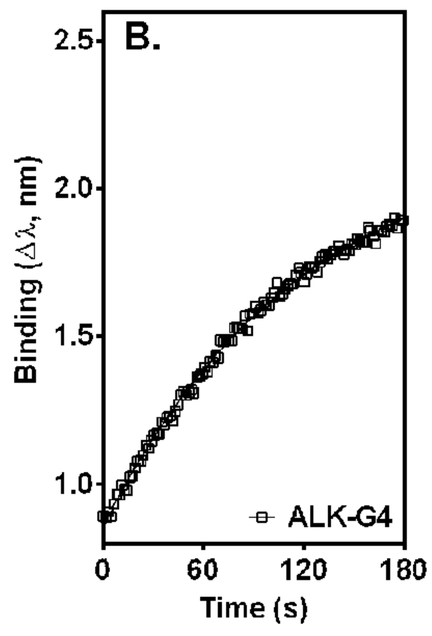



FIG. 18B

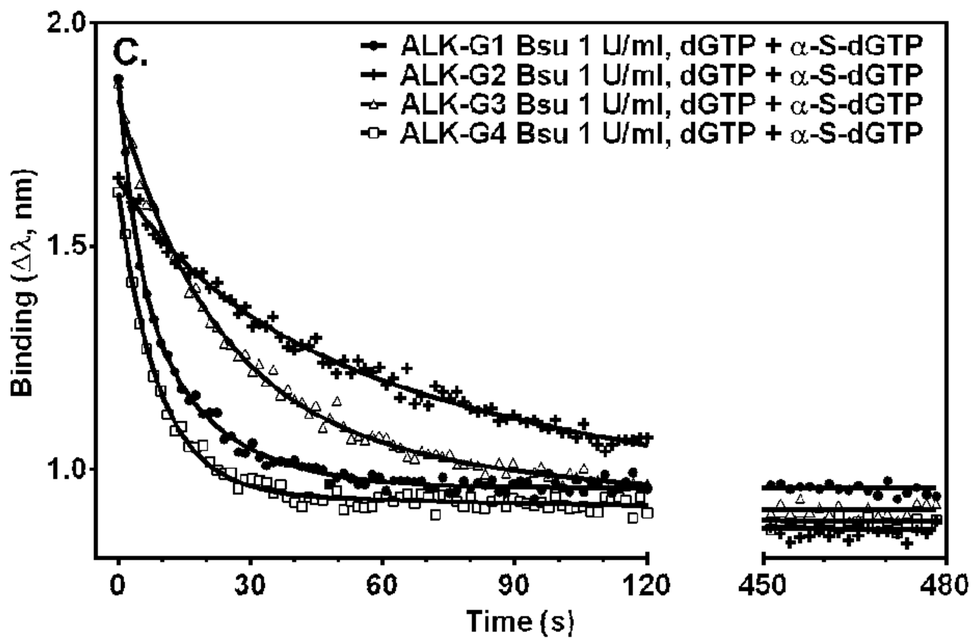



FIG. 18C

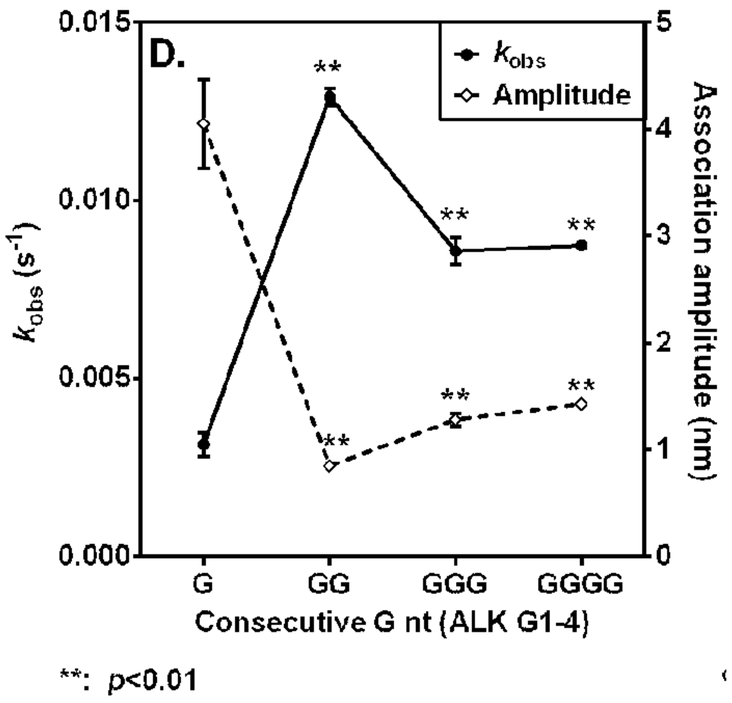



FIG. 18D

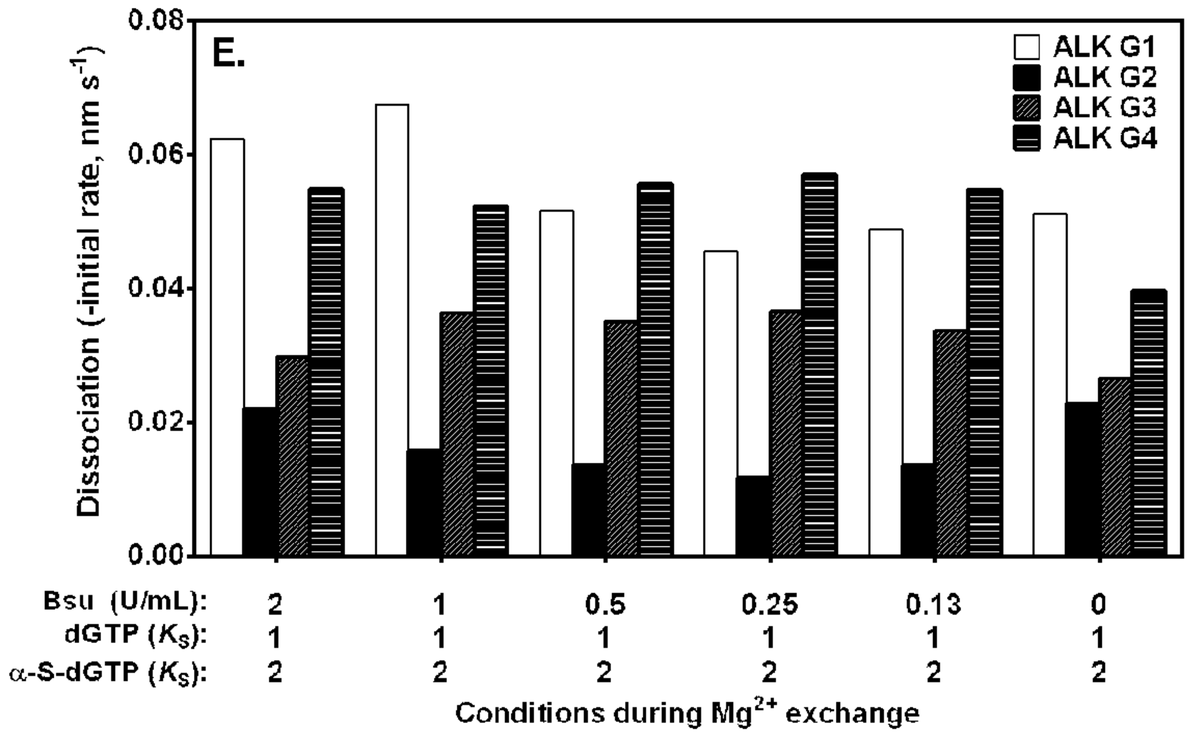



FIG. 18E

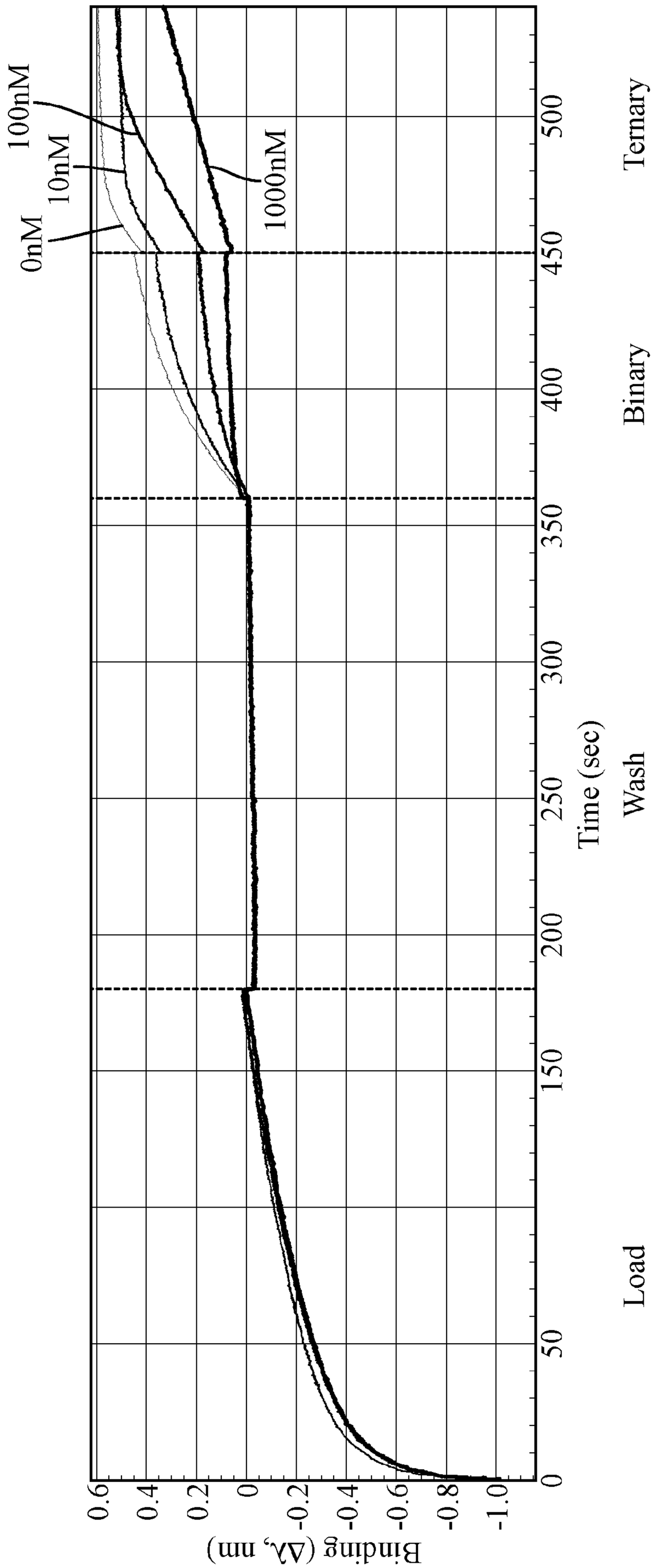

100nM
10nM
0nM
1000nM
Ternary
Binary
Wash
Load
Time (sec)
Binding (Δλ, nm)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
0
50
150
200
250
300
350
400
450
500

FIG. 19

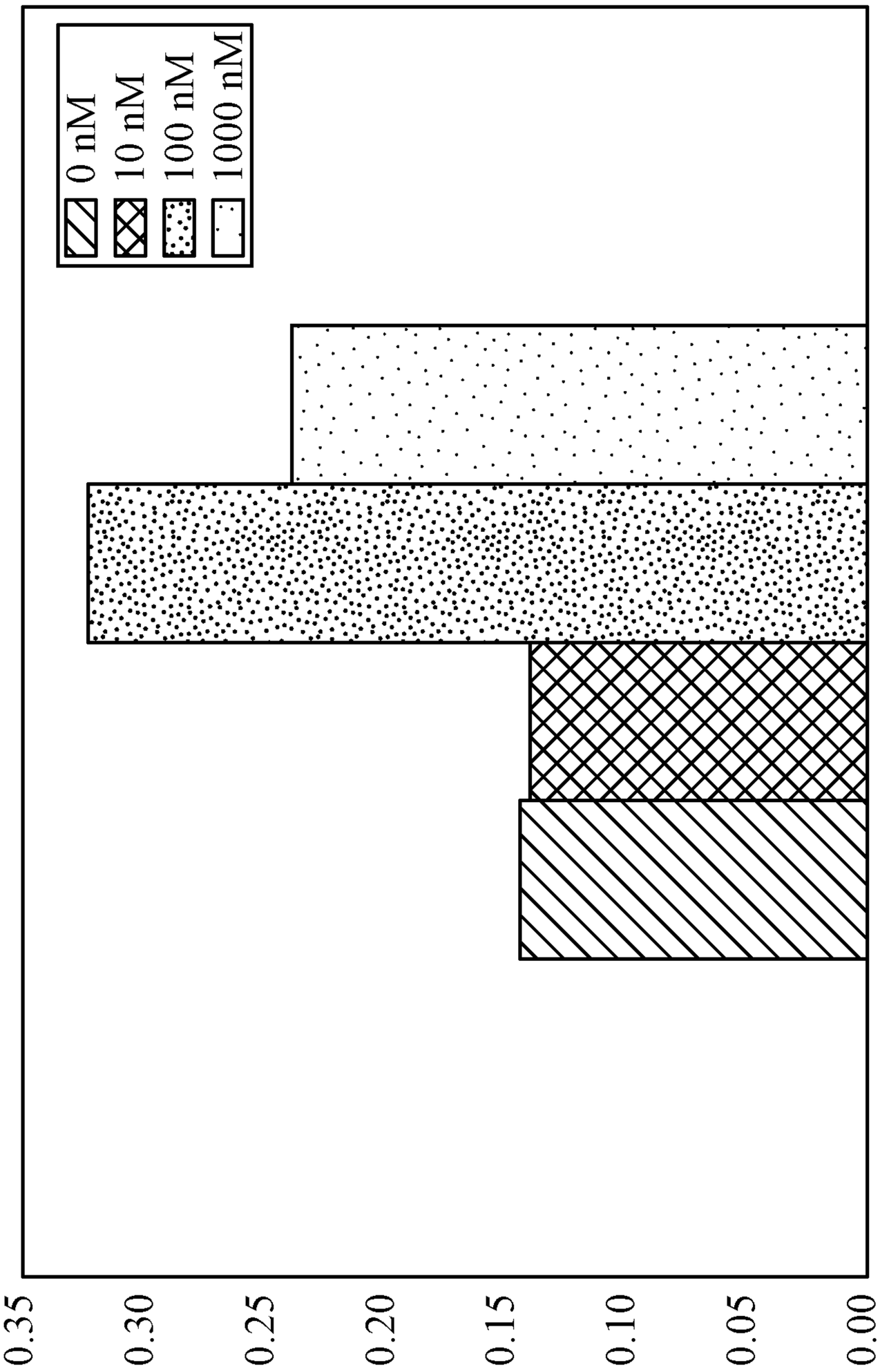

0 nM
10 nM
100 nM
1000 nM
Concentrations of Non-Immobilized Primed Template Nucleic Acid
Binding (Δλ, nm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00

FIG. 20

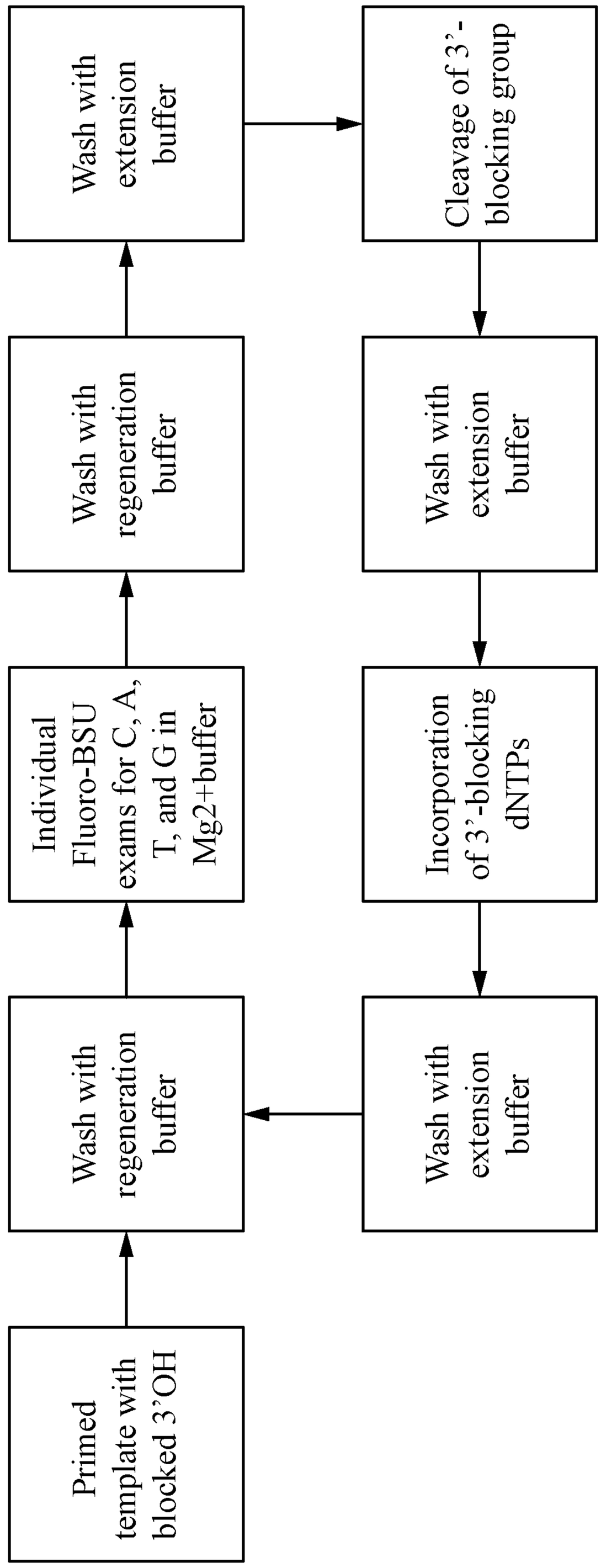

Primed template with blocked 3'OH
Wash with regeneration buffer
Individual Fluoro-BSU exams for C, A, T, and G in Mg2+buffer
Wash with regeneration buffer
Wash with extension buffer
Incorporation of 3'-blocking dNTPs
Wash with extension buffer
Cleavage of 3'-blocking group
Wash with extension buffer
Wash with regeneration buffer

FIG. 21

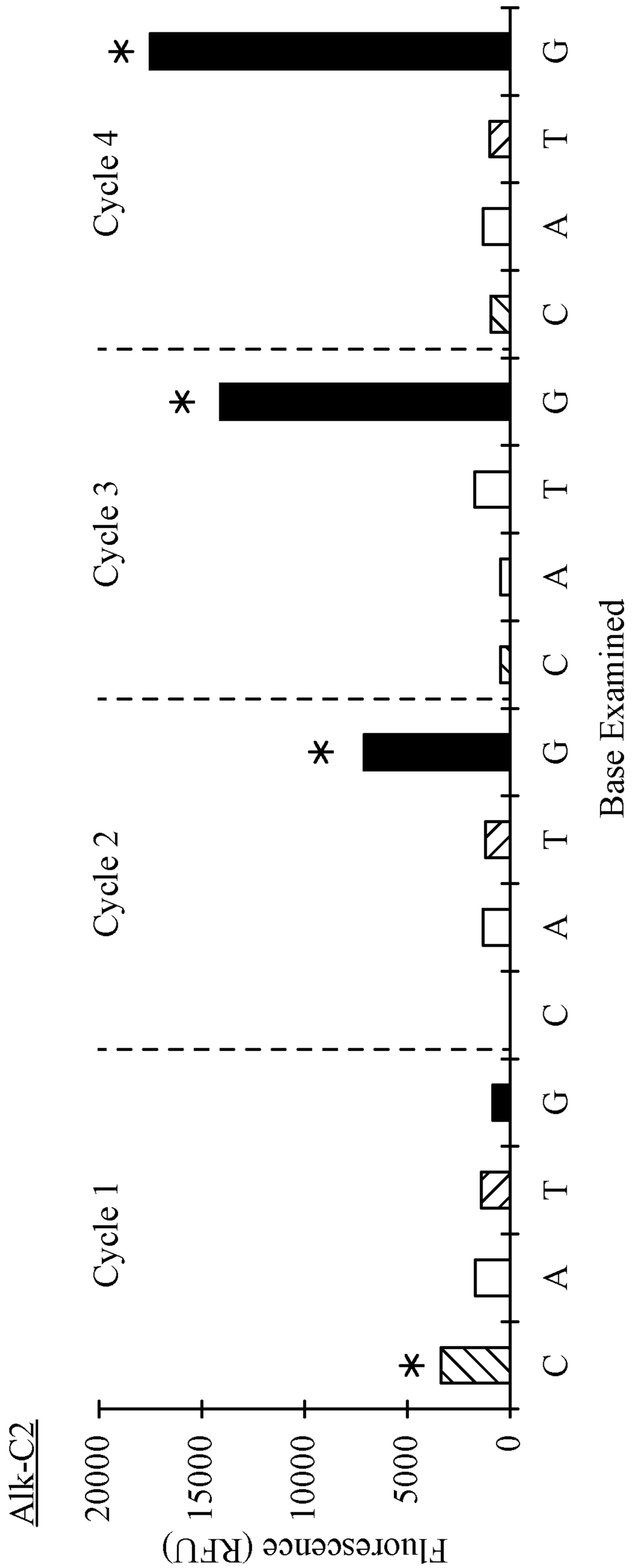

Alk-C2
Fluorescence (RFU)
20000
15000
10000
5000
0
Cycle 1
Cycle 2
Cycle 3
Cycle 4
C A T G C A T G C A T G
Base Examined
*

FIG. 22