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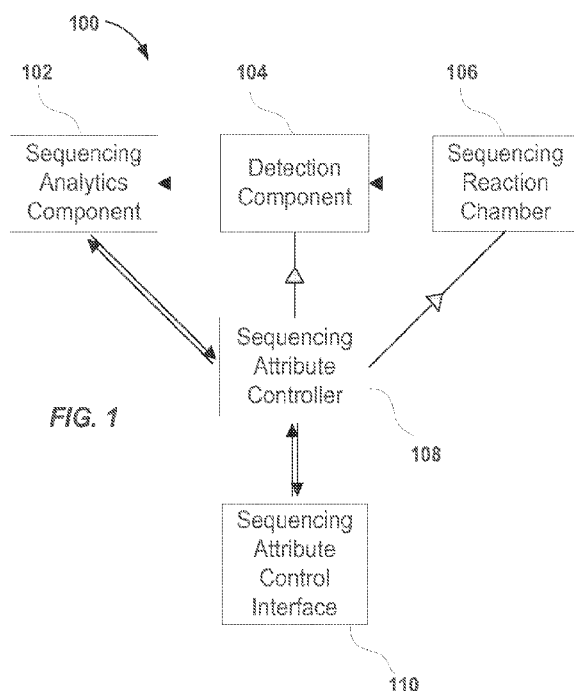
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(54) Title: SYSTEMS AND METHODS FOR ADAPTIVE RECURSIVE SEQUENCING



(57) Abstract: A recursive sequencing system having a sequencing reaction chamber, a detection component, a sequencing analytics component and a sequencing attribute controller is disclosed. The sequencing reaction chamber receives an array with at least one immobilized nucleic acid target. The detection component is interfaced to the sequencing reaction chamber and detects incorporation of one or more fluorescent labeled nucleotides into the nucleic acid targets. The sequencing analytics component is in communication with the detection component. The sequencing attribute controller is in communication with the sequencing reaction chamber, the detection component and the sequencing analytics component. The sequencing attribute controller controls one or more sequencing attributes of the recursive sequencing system.



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SYSTEMS AND METHODS FOR ADAPTIVE RECURSIVE SEQUENCING**CORRESPONDING APPLICATION(S)**

[0001] This application claims benefit of U.S. Application No. 61/307,747, filed on February 24, 2010.

[0002] This application is a continuation-in-part of PCT/US2010/50406, filed on September 27, 2010, which is a continuation-in-part of U.S. Application No. 12/748,168, filed on March 26, 2010, which claims the benefit of U.S. Provisional Application Nos.: 61/164,324, filed on March 27, 2009; 61/184,770, filed on June 5, 2009; 61/242,771, filed on September 15, 2009; 61/245,457, filed on September 24, 2009; 61/263,974, filed on November 24, 2009; 61/289,388; filed on December 22, 2009; 61/293,618, filed on January 8, 2010; 61/293,616, filed on January 8, 2010; 61/299,919, filed on January 29, 2010; 61/299,917, filed on January 29, 2010; 61/307,356, filed on February 23, 2010. All of the aforementioned patent applications are incorporated by reference in their entireties.

FIELD

[0003] The disclosure generally relates to compositions, systems and methods for single molecule sequencing using signals emitted from an energy transfer technique which permits detection and monitoring of nucleotide binding and nucleotide incorporation events. More specifically, the disclosure relates to systems and methods of adaptive recursive sequencing of nucleic acids.

BACKGROUND

[0004] Obtaining nucleic acid sequence information is an important starting point for medical and academic research endeavors. The sequence information facilitates medical studies of active disease, provides for genetic disease predispositions testing, and assists in rational design of drugs targeting specific diseases. Sequence information is also the basis for genomic and evolutionary studies and many genetic engineering applications. In addition, reliable sequence information is useful for paternity tests, criminal investigations and forensic studies.

[0005] Nucleic acid sequence information is typically obtained using chain termination and size separation procedures, such as those described by Sanger, et al., (1977 Proc. Nat. Acad. Sci. USA 74:5463-5467). Prior to gel separation, nucleic acid target molecules are cloned, amplified and isolated. Then, sequencing reactions are conducted in four separate reaction vessels, one for each nucleotide: A, G, C and T. These sequencing methods are adequate for read lengths of 500-10000 nucleotides. However, such methods are time-consuming and require relatively large amounts of target molecules. Additionally, these methods can be expensive, as they require reagents for four reaction vessels. The amplification steps are also error-prone which can jeopardize acquiring reliable sequence information. Furthermore, these methods suffer from sequence-dependent artifacts including band compression during size separation.

[0006] The technological advances in automated sequencing machines, fluorescently-labeled nucleotides, and detector systems have improved the read lengths and permit massively parallel sequencing cycles for high throughput methods. But these procedures are still inadequate for large projects, such as sequencing the human genome. The human genome contains approximately three billion bases of DNA sequence. Procedures that can sequence and analyze the human genome (or the genome of any organism) in a relatively short time span and at a reduced cost may make it feasible to deliver genomic information as part of a healthcare program which can prevent, diagnose, and treat disease.

[0007] Sequencing systems and methods that use energy transfer sequencing techniques overcome many problems associated with current nucleotide incorporation procedures. The energy transfer sequencing techniques utilize minute amounts of target molecules with no amplification steps. The techniques may not use four separate nucleotide incorporation reactions, and the reactions are not size separated or loaded on a gel. In short, energy transfer sequencing techniques facilitate rapid, accurate, and real-time sequencing of long nucleic acid fragments.

DETAILED DESCRIPTION OF THE DRAWINGS

[0008] The embodiments of the disclosure will be illustrated with reference to the following exemplary and non-limiting drawings, in which like elements are numbered similarly, and where:

[0009] FIG. 1 is a schematic representation of an adaptive recursive sequencing system architecture, in accordance with one embodiment of the disclosure;

[0010] FIG. 2 is an exemplary process flowchart of an adaptive recursive sequencing;

[0011] FIG. 3 is a sequencing workflow diagram for an adaptive recursive sequencing system in accordance with one embodiment;

[0012] FIG. 4 depicts one embodiment using an immobilized target molecule/primer duplex to re-sequence the same target molecule, in a direction away from the solid surface, using the reagent exchange methods;

[0013] FIG. 5 depicts another embodiment using an immobilized target molecule/primer duplex to re-sequence the same target molecule, in a direction away from solid surface, using the reagent exchange methods;

[0014] FIG. 6 depicts another embodiment using an immobilized, self-primed target molecule to re-sequence the same target molecule, in a direction away from the solid surface, using the reagent exchange methods;

[0015] FIG. 7 depicts one embodiment using an immobilized target molecule/primer duplex to synthesize an extension product, where the same extension product is re-sequenced in a direction towards the solid surface, using the reagent exchange methods;

[0016] FIG. 8 depicts another embodiment using an immobilized target molecule/primer duplex to synthesize an extension product, where the same extension product is re-sequenced in a direction towards the solid surface, using the reagent exchange methods;

[0017] FIG. 9 depicts one embodiment using an immobilized circular target nucleic acid molecule and a primer for rolling circle replication to re-sequence the same target molecule multiple times;

[0018] FIG. 10 depicts one embodiment using an immobilized double-stranded target nucleic acid molecule, which is ligated at both ends with adaptors, for rolling circle replication to re-sequence the same target molecule multiple times;

[0019] FIG. 11 depicts a graph of branching ratio and deletion probability of different nucleotides under different conditions;

[0020] FIG. 12 is a graph illustrating event duration for nucleotide incorporation under different conditions;

[0021] FIG. 13 and FIG. 14 include graphs of incorporation speed under different conditions; and

[0022] FIG. 15 includes a graph of branch ratio for particular nucleotides under different conditions.

DETAILED DESCRIPTION

[0023] In an embodiment, a sequencing system is disclosed that can allow for the controlled re-sequencing of at least a portion of the same nucleic acid molecule using the same or different sequencing conditions or parameters. During a sequencing cycle, sequencing errors can occur, such as deletions, branching, or misreads. Further, misreads of fluorescent signals, differences in rates of incorporation, undesired cleaving of fluorescent labels prior to nucleotide incorporation, loss of polymerase activity, or other events can lead to errors in sequencing data. Such errors can be expressed in terms of branch ratio, deletion rates, error rates, or accuracy. A sequencing system that gives the user the flexibility to vary one or more sequencing conditions or parameters during or between each sequencing cycle allows for an overall reduction in base read error rates and potentially allows the sequencing system to be used for a host of different genome sequencing applications, including, but not limited to methylome sequencing or de novo sequencing, among others. In particular, the system may control conditions or parameters, such as pH, temperature, ionic strength, types of ions, variations or analogs of labeled nucleotides, types of polymerases or energy transfer donor moieties, or any combination thereof. Such control may be performed in response to determining a performance metric, such as branch ratio, deletion rates, error rates, or accuracy of a sequencing cycle. By performing multiple sequencings under different conditions, the effect of random errors and systematic errors, such as errors associated with incorporation of specific nucleotides, can be diminished.

[0024] The disclosure generally relates to compositions, systems and methods for single molecule sequencing using fluorescent signals emitted from an energy transfer

sequencing technique, which permits detection and monitoring of nucleotide binding and nucleotide incorporation events. Specifically, the disclosure relates to systems and methods of adaptive recursive sequencing of nucleic acids.

[0025] In an aspect, the disclosure relates to a recursive sequencing system having one or more of a sequencing reaction chamber, a detection component, a sequencing analytics component or a sequencing attribute controller. The sequencing reaction chamber receives an array with an immobilized nucleic acid target. The detection component is interfaced to the sequencing reaction chamber and detects incorporation of one or more fluorescent labeled nucleotides into the nucleic acid target. The sequencing analytics component is in communication with the detection component. The sequencing attribute controller is in communication with the sequencing reaction chamber, the detection component and the sequencing analytics component. The sequencing attribute controller controls one or more sequencing attributes or hardware attributes of the recursive sequencing system.

[0026] In another aspect, a method for adaptive recursive sequencing is disclosed. The method includes selecting a first sequencing attribute for sequencing a target nucleic acid; supplying a first reaction mixture to a reaction chamber containing the target nucleic acid based on the selected first sequencing attribute, the first reaction mixture comprising a plurality of labeled nucleotides, a first polymerase and a first energy transfer donor moiety; detecting the incorporation of at least one of the plurality of labeled nucleotides to the target nucleic acid; removing any incorporated nucleotide from the target nucleic acid; selecting a second sequencing attribute for sequencing the target nucleic acid; supplying a second reaction mixture to a reaction chamber based on the selected second sequencing attribute, the second reaction mixture comprising a plurality of labeled nucleotides, a second polymerase and a second energy transfer donor moiety; and detecting the incorporation of at least one of the plurality of labeled nucleotides to the target nucleic acid.

[0027] The systems and methods provided herein use sequence-by-synthesis procedures for deducing the sequence of a nucleic acid target molecule. The methods permit detection and monitoring of nucleotide binding and nucleotide incorporation events. In an example, polymerases are attached with an energy transfer donor moiety and nucleotide polyphosphate molecules are attached with at least one energy transfer acceptor moiety. The donor and acceptor moieties undergo energy transfer

when the polymerase and nucleotide are proximal to each other during nucleotide binding or nucleotide incorporation. As the donor and acceptor moieties undergo energy transfer, they emit a signal (or a change in a signal) which may correlate with nucleotide binding or incorporation. Detecting a time sequence of the emitted signals, or the change in the emitted signals, can be used to determine the order of the nucleotide incorporation, and can therefore be used to deduce the sequence of the target molecule.

[0028] Adaptive recursive sequencing allows for the customization of sequencing reaction conditions (e.g., detector type, filters, reaction chamber parameters, etc.) and sequencing reaction reagent mixtures (e.g., polymerases, electron acceptor moieties, electron donor moieties, catalysts, etc.) during the repeated sequencing (i.e., recursive sequencing) of a nucleic acid target in a sequencing reaction chamber. Such conditions and reagent mixtures can be controlled to provide desirable branch ratios, low deletion rates, or improved accuracy. Branch ratio is the ratio of productive events (i.e., a nucleotide is incorporated) to all events. In particular instances, a nucleotide that leaves without binding can fluoresce, providing a false incorporation signal.

[0029] FIG. 1 is a schematic representation of an adaptive recursive sequencing system architecture, in accordance with one embodiment of the disclosure. The adaptive recursive sequencing system 100 includes a sequencing analytics component 102, a detection component 104, a sequencing reaction chamber 106, a sequencing attribute controller 108 and a sequencing attribute control interface 110.

[0030] In an embodiment, the sequencing reaction chamber 106 accommodates one or more nucleic acid target arrays that include a plurality of nucleic acid target molecules. The nucleic acid target molecules are immobilized onto a surface and sequenced via sequencing-by-synthesis (i.e., polynucleotide polymerization) reactions. The sequencing reactions are carried out by removable or replaceable sequencing engines. Each of the sequencing engines includes a polymerase attached to an energy transfer moiety, such as an energy transfer donor moiety.

[0031] The energy transfer donor moiety is capable of absorbing electromagnetic energy (e.g., light) at a first wavelength and emitting excitation energy in response. In one embodiment, the energy transfer donor moiety is a fluorescence resonance energy

transfer (“FRET”) capable nanocrystal. In another embodiment, the energy transfer donor moiety is a FRET capable dye molecule.

[0032] Before a nucleic acid target molecule is sequenced, a primer polynucleotide molecule having a 3' OH terminus (the terminal 3' OH provides the polymerization initiation site for DNA polymerase) can be attached to a portion of the nucleic acid target molecule via complementary based pairing. The sequencing engine then binds to the 3' OH terminus and incorporates a nucleotide candidate that is complementary with the closest non-paired template nucleotide on the nucleic acid target molecule.

[0033] Conventionally, an energy transfer acceptor moiety is operably linked to each of the nucleotide candidates that are interrogated by the polymerase for complementarity. The nucleotide polyphosphate molecules can be labeled in a way that does not interfere with the events of polymerization. For example, the attached energy transfer acceptor moiety does not interfere with nucleotide incorporation, does not interfere with cleavage of the phosphodiester bonds, or does not interfere with release of the polyphosphate product.

[0034] The energy transfer acceptor moiety is capable of absorbing excitation energy emitted by a donor and fluorescing at a second wavelength. Thus, different nucleotide polyphosphate molecules (*e.g.*, adenosine, thymidine, cytidine, guanosine, and uridine) can be labeled with different energy transfer acceptor moieties so that the detectable signals from each of the different nucleotide polyphosphate molecules can be distinguishable to permit base identity.

[0035] Once an energy transfer acceptor labeled nucleotide is incorporated into the nucleic acid target molecule by the polymerase, the donor and acceptor moieties can interact with each other in a manner which produces a detectable signal (*e.g.*, via FRET). Such a signal can be detected by the detection component 104. The detection component 104 is interfaced to the sequencing reaction chamber 106.

[0036] In an embodiment, the energy transfer acceptor moiety may be operably linked to any position of the nucleotide polyphosphate molecule or linked to any phosphate group, the sugar or the base. For example, the energy transfer acceptor moiety can be operably linked to the terminal phosphate group. In another embodiment, the nucleotide polyphosphate molecule may be operably linked with an additional energy transfer acceptor moiety so that the nucleotide polyphosphate molecule is attached with two or more energy transfer acceptor moieties. The

additional energy transfer acceptor moiety can be the same or different than the first energy transfer acceptor moiety.

[0037] In another embodiment, the nucleotide polyphosphate molecules comprise a sugar moiety, base moiety and at least three or more phosphate groups linked to the sugar moiety by an ester or phosphoramidate linkage. The phosphates can be linked to the 3' or 5' C of the sugar moiety. The nucleotide polyphosphate molecules can be incorporated or polymerized into a growing nucleic acid strand by a naturally occurring, modified or engineered nucleic acid dependent polymerase.

[0038] The detection component 104 is interfaced with the sequencing reaction chamber 106 to allow the detection component 104 to register light emissions from the energy transfer acceptor moiety during nucleotide incorporation. That is, the detection component 104 is configured to detect light emissions resulting from one or more nucleotide incorporation events occurring within the sequencing reaction chamber 106. The detection component 104 can include one or more optical elements (*e.g.*, prisms, diffraction elements, lenses, filters, objective trains, etc.) and detection elements (*e.g.*, CCD, CMOS, laser wavelengths, etc.).

[0039] The detection component 104 is communicatively connected to the sequencing analytics component 102, which houses or stores the various hardware and software elements to process and analyze the light emission signals that are captured by the detection component 104. For example, the detection component 104 can include an optical detection component, such as a charge coupled device (CCD) or a CMOS imaging device. In addition, the detection component 104 can include optics components and excitation sources. The sequencing analytics component 102 can include an image processing element, a signal processing element, a genome mapping and assembly element and a bioinformatics element. The various processing elements of the sequencing analytics component 102 can analyze the signals registered by the detection component 104 to make base calls, assemble the base reads from the various segments of a sample nucleic acid laid out onto the sequencing arrays into a complete nucleotide sequence and provide one or more quality metrics (*e.g.*, performance metrics) about the sequencing system. Exemplary performance metrics include branch ratio, deletion rate, error rate, or accuracy, among others. The quality metrics can provide an indication of systematic or random errors that occur during a set of sequencing cycles.

[0040] A sequencing attribute controller 108 communicates with the sequencing analytics component 102, detection component 104, sequencing reaction chamber 106 and sequencing attribute control interface 110. In embodiments, the sequencing attribute controller 108 can be a standard electronic control unit that includes control circuitry for providing control functions, input interface circuitry for receiving input signals from external devices, output interface circuitry for providing output signals for controlling the functions or operations of one or more connected devices. In other embodiments, the sequencing attribute controller 108 can be a software program or engine that can be stored as non-transitory computer readable code on a tangible computer readable medium (*e.g.*, hard drives, network attached storage (NAS), read-only memory, random-access memory, CD-ROMs, CD-Rs, CD-RWs, magnetic tapes, optical data storage devices, etc.) that is part of a standalone computing system or device or a part of the sequencing analytics component 102 or the sequencing attribute control interface 110.

[0041] The sequencing attribute controller 108 provides control instructions to change or modify one or more sequencing attributes of the detection component 104 or the sequencing reaction chamber 106. The sequencing attribute controller 108 receives control instructions from the sequencing analytics component 102 or the sequencing attribute control interface 110. Sequencing attributes may include one or more components of the sequencing reaction mix (*i.e.*, reagent exchange of any chemical or biological component of the sequencing reaction, etc.) or sequencing hardware component (*e.g.*, detector type, filter type, reaction chamber conditions, etc.). Some examples of sequencing attributes that can be changed or modified during a sequencing cycle or in between sequencing cycles with the same nucleic acid target include, but are not limited to, pH, temperature, ionic strength, types of ions, variations and analogs of labeled nucleotides, variations in types of polymerases or energy transfer donor moieties, or any combination thereof.

[0042] For example, the sequencing attribute may be a reagent exchange during or between sequencing cycles. Reagents which can be exchanged include any reagent which is used in a nucleotide binding or nucleotide incorporation reaction, including but not limited to any type of: polymerase; energy transfer moiety associated with the polymerase; nucleotides (*e.g.*, hydrolyzable, non-hydrolyzable, chain-terminating, or labeled or non-labeled nucleotides); the synthesized strand; compounds which reduce

photo-damage; buffers; salts; co-factors; divalent cations; chelating agents; or any combination thereof. In an example, the type of polymerase can be changed during a cycle or between cycles. Different polymerases provide different rates of incorporation, different tolerances of repeat segments, different tolerances of particular dyes and labeled nucleotides, or different interactions with ions. For example, processive polymerase such as Phi29 can be used in one sequencing cycle and distributive polymerase in a different sequencing cycle. Other examples of polymerases that may be swapped include, Klenow, Taq, T7 SEQUENASE™ (GE), TAQ FS™ (ABI), RB69, and any other polymerase compatible with the system.

[0043] Variations and analogs of nucleotides can be changed during a sequencing cycle or between cycles. Nucleotides having analog bases, analog sugars, differences in number of phosphate groups, bridging oxygen substitutions, side chain oxygen substitutions, different linkers, different dye pairings, or any combination thereof, can be exchanged during or between sequencing cycles.

[0044] For example, the pairing of dyes with nucleotides can be changed between cycles. Particular combinations of nucleotides and dyes may provide higher error rates or result in branching or deletions. In an example, one round of a reagent exchange reaction can be conducted using three types of nucleotides (e.g., A, G, and C) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., T) can be unlabeled. In one embodiment, the A nucleotides can be labeled with dye type 1, G nucleotides can be labeled with dye type 2, and C nucleotides can be labeled with dye type 3. In a second round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., G, C, and T) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., A) can be unlabeled. In a third round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., C, T, and A) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., G) can be unlabeled. In a fourth round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., T, A, and G) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., C) can be unlabeled. The first, second, third, and fourth rounds of reagent exchange reactions can be conducted in any order, and in any combination. In any of the rounds

of reagent exchange reactions, the different types of nucleotides can be linked to the same or different types of energy transfer dye.

[0045] In another example, multiple rounds of reagent exchange reactions can be conducted using four types of nucleotides (e.g., A, G, C, and T) each labeled with a different type of energy transfer acceptor dye in each round. In one embodiment, in round one, the A nucleotides can be labeled with dye type 1, G labeled with dye type 2, C labeled with dye type 3, and T labeled with dye type 4. In a subsequent round, the reagent exchange reaction can be conducted using A labeled with dye type 2, G labeled with dye type 3, C labeled with dye type 4, and T labeled with dye type 1.

[0046] In a particular example, nucleotide-dye pairs can be changed between sequencing cycles of the same nucleic acid target. That is, in a first cycle, fluoracein may be the acceptor dye associated with adenosine (A) incorporation. In a different cycle, A may be associated with Texas Red. Nucleotide pairs can be changed between cycles without limitation as long as they are compatible with the sequencing system and are distinguishable from the other nucleotide-dye pairs. For example, in a first sequencing cycle the nucleotide acceptor dye set can be adenosine (A)-FAM, cytidine (C)-R6G, guanosine (G)-TMR, thymidine (T)-ROX. In the second sequencing cycle, the nucleotide acceptor dye combination can be changed to A-AF635, C-AF660, G-AF700, and T-AF750. In the third sequencing cycle, the dye nucleotide combination can again be modified to be A-R6G, C-TMR, G-ROX, T-FAM. In the fourth sequencing cycle, a dye may be missing from one of the nucleotides, giving a distinct kind of data particular to that cycle.

[0047] Further, the sequencing attribute can include conditions, such as pH, temperature, ion concentration, or ionic strength. For example, in embodiments, pH can be changed in between cycles. That is, in a first cycle the pH may be 7.0 and in the subsequent cycle the pH may be 6.5. Typically, the pH range can be from about 6 to about 7.5, about 5 to about 8 or about 3 to about 11.

[0048] In embodiments, the divalent cations included in the reaction mixture can be changed between cycles. For example, in a first cycle the divalent cation can be Mn^{++} and in a subsequent cycle it can be Mg^{++} . In a further example, Ca^{2+} can be included. In still another cycle the reaction mixture can include a blend of 2 or more different cations. Such incorporation can alter the incorporation rate associated with a polymerase, slowing or accelerating nucleotide incorporation. In embodiments, the

concentration of the cations included in the mixture can be changed between sequencing cycles and can range from about 1 nM to about 100 mM.

[0049] Ionic strength may also be changed. In some embodiments, detergents may also be added or changed. It should be appreciated that essentially any sequencing attribute (*e.g.*, salts, solvents, carbohydrates, fats, nucleotides, proteins, cofactors, and any other component that is compatible with the system) can be changed or modified within or between a sequencing cycles using the recursive sequencing system 100 disclosed herein. Non-exclusive examples of proteins that may be added are single stranded DNA binding protein, double stranded DNA binding protein, Sso7D, bovine serum albumin, or any other protein compatible with the system.

[0050] The nucleic acid target can be changed between sequencing cycles. An example of the target being modified is the newly synthesized strand being removed between sequencing cycles. Removal can be accomplished by treatment with base, acid, salt, solvents, exonucleases or combinations thereof.

[0051] Another example of the nucleic acid being modified between sequencing cycles is the deamination of the target for methylome identification type applications. Deamination is a technique of modifying the target between sequencing reaction cycles to help identify the methylation state of a target. There are various methods to deaminate nucleic acids, including, but not limited to treating the nucleic acid with bisulfite, metabisulfate, nitrous acid, or hydroxylamine, among other agents. In an embodiment, the sequence of a nucleic acid target is first analyzed in one sequencing cycle. The nucleic acid target can be treated with a methylation state distinguishing reagent such as bisulfite. Methylated sites on the template such methylC or hydroxymethylC are protected from deamination by bisulfite. Non-methylated DNA is not protected and C is deaminated to form U. The nucleic acid target is then re-sequenced in a subsequent sequencing reaction cycle. Analysis comparing the two sequencing cycle results can be used to determine the sites of methylation.

[0052] Some examples of other sequencing hardware component attributes that can be changed or modified during a sequencing cycle or between sequencing cycles with the same nucleic acid target include, but are not limited to excitation characteristics (*e.g.*, laser wavelength, laser intensity, etc.), excitation modulation schemes (*e.g.*, continuous, pulsed, amplitude modulated, etc.), excitation modalities (*e.g.*, epi, objective TIRF, prism TIRF, waveguide TIRF, etc.), detection modalities (*e.g.*,

camera-based imaging, detector arrays, FLIM gated imaging, etc.), detector types (e.g., CCD, CMOS, APD arrays, etc.), detection element settings (e.g., signal amplification gain, camera pixel depth, frame rate, etc.), laser types, reaction chamber temperature or pressure conditions (e.g., hydrodynamic flow characteristics, etc.), or any combination thereof.

[0053] Returning to FIG. 1, the sequencing attribute control interface 110 can be a manual or an electronic control interface (e.g., keyboard, dial, knob, switch, button, desktop computer, laptop, wireless device, etc.) that is configured to allow a user to manually input one or more sequencing attributes for the sequencing attribute controller 108 to relay to the detection component 104 or sequencing reaction chamber 106. The sequencing attributes may be application specific (e.g., methylation detection, de novo sequencing, etc.) or related to improving the performance metrics of the sequencing system (e.g., overall error rate of the sequencing system).

[0054] In embodiments, the sequencing attribute controller 108 can receive sequencing attribute inputs automatically from the sequencing analytics component 102 to correct for one or more performance metrics (e.g., error rate, etc.) of the sequencing system (e.g., to account for systematic or random errors). That is, the sequencing analytics component 102 can automatically provide sequencing attribute instructions to the sequencing attribute controller 108 to reduce systematic or random errors in the sequencing system 100. The sequencing attribute controller 108 can change a sequencing attribute or hardware attribute in response to the performance metric.

[0055] The sequencing attributes can be changed or modified in the middle of, or during, the repeated sequencing (i.e., recursive sequencing) of a nucleic acid target. That is, the attribute change can be during a sequencing cycle (i.e., before the sequencing of a target is complete) or between sequencing cycles (i.e., after the sequencing of a target is complete).

[0056] FIG. 2 includes an exemplary process flowchart of an adaptive recursive sequencing. At 202, a first sequencing attribute is selected for sequencing a target nucleic acid. At 204, a second sequencing attribute can be optionally selected for sequencing the target nucleic acid. At 206, a detection component or reaction chamber parameter can be modified based on the selected second sequencing

attribute. In particular, the second sequencing attribute can be selected based on detected incorporation events and associated performance metrics, such as error rates or accuracy.

[0057] At 208, a first reaction mixture is supplied to the reaction chamber containing the target nucleic acid based on the selected first sequencing attribute. The first reaction mixture includes a plurality of labeled nucleotides, a first polymerase and a first energy transfer donor moiety. In an embodiment, the energy transfer donor moiety is a FRET capable nanocrystal. In another embodiment, the energy transfer donor moiety is a FRET capable dye molecule. At 210, the incorporation of at least one of the plurality of labeled nucleotides to the target nucleic acid is detected.

[0058] At 212, the incorporated nucleotide is removed from the target nucleic acid. At 214, a third sequencing attribute for sequencing the target nucleic acid is selected. At 216, a fourth sequencing attribute can be optionally selected for sequencing the target nucleic acid. At 218, a detection component or reaction chamber parameter can be modified based on the selected fourth sequencing attribute. At 220, a second reaction mixture is supplied to the reaction chamber containing the target nucleic acid based on the selected third sequencing attribute. The second reaction mixture includes a plurality of labeled nucleotides, a first polymerase and a second energy transfer donor moiety. At 222, the incorporation of at least one of the plurality of labeled nucleotides to the target nucleic acid is detected. It should be understood, that such a sequence of steps can be repeated to sequence a single nucleic acid target using the same set or a different set of sequencing attributes. In particular, for each sequencing cycle, attributes can be selected based on performance metrics associated with prior sequencing cycles. Such selection can be performed using heuristics or model-based algorithms for adjusting attributes in response to performance metrics.

[0059] FIG. 3 is a sequencing workflow diagram for an adaptive recursive sequencing system in accordance with an embodiment. A user can utilize a sequencing attribute control interface 110 to provide user selected attributes for sequencing a target nucleic acid, as illustrated at 302. A sequencing attribute can be either a reaction mixture attribute, a hardware attribute, or a combination thereof. For example, when a different energy transfer donor is selected, a different excitation frequency may be selected.

[0060] When a reaction mixture attribute is selected at 304, each reaction mixture attribute corresponds to a particular reaction mixture component (306) that is supplied to the reaction chamber to sequence a target nucleic acid, as illustrated at 308. That is, when a reaction mixture attribute is changed, one or more sequencing reaction components are correspondingly modified or changed. For example, when a reaction mixture attribute is changed, the type of polymerase, reporter dye, energy transfer donor moiety (e.g., a nanocrystal), nucleotide, reaction catalyst or adjuvant supplied to the reaction chamber is changed.

[0061] When a hardware attribute is selected at 304, each hardware attribute corresponds to a hardware component type, hardware component operational setting or reaction chamber sequencing parameter to sequence a target nucleic acid, as illustrated at 310. For example, when a hardware attribute is changed, the excitation characteristic (e.g., laser wavelength, laser intensity, etc.), excitation modulation scheme (e.g., continuous, pulsed, amplitude modulated, etc.), excitation modality (e.g., epi, objective TIRF, prism TIRF, waveguide TIRF, etc.), detection modality (e.g., camera-based imaging, detector arrays, FLIM gated imaging, etc.), detector type (e.g., CCD, CMOS, APD arrays, etc.), detection element setting (e.g., signal amplification gain, camera pixel depth, frame rate, etc.), laser type, reaction chamber temperature or pressure condition (e.g., hydrodynamic flow characteristics, etc.) used in the sequencing of the target nucleic acid is changed.

[0062] Once the reaction mix and hardware attributes have been selected and implemented, as illustrated at 308 and 310, the target nucleic acid is sequenced in the reaction chamber, as illustrated at 314. A detection component interfaced with the reaction chamber detects the light emitted by the electron transfer donor/acceptor moieties during a nucleotide incorporation event and directs data to a sequencing analytics component 102 for processing and analysis, as illustrated at 316. The sequencing analytics component 102 can then optionally provide sequencing attribute inputs based on a read error correction analysis to minimize or eliminate system or random base call error rates, as illustrated at 318.

[0063] FIG. 4 depicts an embodiment using an immobilized target molecule/primer duplex to re-sequence the same target molecule, in a direction away from the solid surface, using the reagent exchange methods.

[0064] The system disclosed in FIG. s 1-3 can be used for re-sequencing at least a portion of the same nucleic acid molecule, replacing any reagent used to practice nucleotide binding or nucleotide incorporation with functional reagents to permit continuing the nucleotide incorporation reaction on the same nucleic acid molecule, or performing nucleotide binding or nucleotide incorporation reactions and switching to reactions having different nucleotide binding or nucleotide incorporation reaction properties on the same nucleic acid molecule.

[0065] The reagents which can be exchanged include any reagent which is used in a nucleotide binding or nucleotide incorporation reaction, including but not limited to any type of: polymerase; energy transfer moiety associated with the polymerase; nucleotides (e.g., hydrolyzable, non-hydrolyzable, chain-terminating, or labeled or non-labeled nucleotides); the synthesized strand; compounds that reduce photo-damage; buffers; salts; co-factors; divalent cations; and chelating agents. The fresh reagents can be the same or different from the old reagents. For example, one round of a reagent exchange reaction can be conducted using three types of nucleotides (e.g., A, G, and C) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., T) can be unlabeled. In one embodiment, the A nucleotides can be labeled with dye type 1, G nucleotides can be labeled with dye type 2, and C nucleotides can be labeled with dye type 3. In a second round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., G, C, and T) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., A) can be unlabeled. In a third round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., C, T, and A) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., G) can be unlabeled. In a fourth round, the reagent exchange reaction can be conducted using three types of nucleotides (e.g., T, A, and G) labeled with a different type of energy transfer acceptor dye, and another different type of nucleotide (e.g., C) can be unlabeled. The first, second, third, and fourth rounds of reagent exchange reactions can be conducted in any order, and in any combination. In any of the rounds of reagent exchange reactions, the different types of nucleotides can be linked to the same or different type of energy transfer dye.

[0066] In an aspect, the reagent exchange methods can be used to sequence the same target nucleic acid molecule 1, 2, 3, 4, or 5 times, or up to 10 times, or up to 25 times,

or up to 50 times, or more than 50 times. For example, errors in detecting or identifying the incorporated nucleotides may be reduced by re-sequencing the same target molecule. The errors can arise when a non-reporting nucleotide (e.g., which is linked to a non-reporting energy transfer acceptor dye) is incorporated but does not emit a detectable signal. The same target molecule can be sequenced one or more times to provide redundant nucleotide sequence information. The reagent exchange methods can be used to sequence the strand which is synthesized during a nucleotide incorporation reaction. The synthesized strand can be sequenced 1, 2, 3, 4, or 5 times, or up to 10 times, or up to 25 times, or up to 50 times, or more than 50 times, to provide redundant nucleotide sequence information. The same target molecule, or synthesized strand, can be re-sequenced using exchanged primers having sequences which are the same or a different from the sequence of the old primers. Sequencing the same target molecule multiple times, or sequencing the same synthesized strand multiple times, can provide multiple data sets of sequence information which can be aligned and compared. In an embodiment, the alignment can be used to deduce a consensus sequence of the target molecule or the synthesized strand. The alignment can be used to provide multi-fold coverage of the nucleotides which are contained within the target molecule or synthesized strand.

[0067] The reagent exchange methods can be used to replace inactive polymerases or non-functional nucleotides or energy transfer moieties, with fresh polymerase (e.g., Phi29 and Klenow or T7), nucleotides, or other reagents, in order to continue the nucleotide incorporation reaction on the same target molecule or synthesized strand. For example, fresh polymerase, nucleotides, or reagents can be added to the immobilized target/primer molecules to permit continuation of the nucleotide incorporation reaction on the same target or synthesized molecule.

[0068] The reagent exchange methods can be used to replace the reagents in an on-going nucleotide binding or incorporation reaction, in order to switch to a different type of nucleotide binding or nucleotide incorporation reaction on the same target or synthesized molecule. For example, the first nucleotide incorporation reaction can be conducted using a polymerase, nucleotides, and other reagents, which exhibit certain properties, such as: nucleotide fidelity; rate of nucleotide incorporation; processivity; strand displacement; kinetics of nucleotide binding, catalysis, release of the cleavage product, or polymerase translocation; exonuclease activity; or activity at certain

temperatures. The reagents (e.g., polymerases or nucleotides) can be exchanged with different reagents to conduct a nucleotide incorporation reaction which exhibits different nucleotide incorporation properties (on the same target molecule or on the same synthesized strand).

[0069] The reagent exchange methods can be practiced using any type of nucleotide binding or nucleotide incorporation reactions, including but not limited to: the energy transfer methods disclosed herein; any type of discontinuous reactions (e.g., synchronous nucleotide incorporation methods described in: (U.S. Serial No. 61/184774, filed on June 5, 2009, docket No. LT00034 PRO; or U.S. Serial No. 61/242762, filed on September 15, 2009, docket No. LT00054 PRO; and U.S. Serial No. 61/180811, filed on May 22, 2009, docket No. LT00033 PRO); and any type of continuous reactions (e.g., asynchronous nucleotide incorporation methods as described in: (PCT/US2010/50406, filed on September 27, 2010; U.S. Application No. 12/748,168, filed on March 26, 2010; U.S. Serial No. 61/077,090, filed on June 30, 2008; U.S. Serial No. 61/089,497, filed on August 15, 2008; U.S. Serial No. 61/090,346, filed on August 20, 2008; PCT application No. PCT/US09/49324, filed on June 30, 2009; U.S. Serial No. 61/164324, filed on March 27, 2009, docket No. LT00019 PRO; and U.S. Serial No. 61/263974, filed on November 24, 2009, docket No. LT00019 PRO2).

[0070] The reagent exchange methods can be practiced using any type of format using an immobilized primer; target molecule; synthesized strand; or polymerase. The reagent exchange methods can be practiced on a single target nucleic acid molecule, or on random or organized arrays of single nucleic acid molecules, and using any type of solid surface (U.S. Serial No. 61/220174, filed on June 24, 2009, docket No. LT00036 PRO; and U.S. Serial No. 61/245248, filed on September 23, 2009, docket No. LT00061 PRO). The target molecules and synthesized strands can be genomic, recombinant, DNA, RNA, double-stranded, or single-stranded nucleic acid molecules. The target nucleic acid molecules can be linear or circular. The target nucleic acid molecules can be self-priming molecules or can be associated with primer molecules. The target nucleic acid molecules can be immobilized using any method, including the methods depicted in FIGs. 4-10.

[0071] Provided herein are reagent exchange methods, where the existing target molecule, synthesized strand, primer, polymerase, nucleotides, or other reagents, can

be removed in a manner which does not remove the immobilized target molecule, primer, or synthesized strand. In some embodiments, the primer, target molecule, or synthesized strand can be removed. Methods for removing the components include physical, chemical, or enzymatic methods.

[0072] The polymerase can be inactivated or removed using physical, chemical, or enzymatic method, in any combination and in any order. For example, the polymerase can be deactivated using elevated temperatures, such as 45-80 °C, for about 30 seconds to 10 minutes. In another example, the polymerase can be removed from the target molecule or synthesized strand using a protein-degrading enzyme, such as proteinase-K. In another example, the polymerase can be removed from the target molecule or synthesized strand using compounds known to disrupt protein complexes, where the compounds include detergents (e.g., N-lauroyl sarcosine, SDS), chaotropic salt (e.g., guanidinium hydrochloride), lithium sulfate, and EDTA.

[0073] Any combination of capture molecule, primer, target molecule, or synthesized strand, can be dissociated (e.g., denatured) from each other using physical, chemical, or enzymatic methods, in any combination and in any order. For example, the target molecule/synthesized strand duplex can be denatured using elevated temperatures, such as about 75-100 °C (e.g., without formamide) or about 45-90 °C (e.g., with formamide). In another example, the target molecule or synthesized strand can be degraded using a nucleic acid degrading enzyme, such as a 5' → 3' or 3' → 5' exonuclease (e.g., exonuclease III, T7 gene 6 exonuclease, exonuclease I). In yet another example, the target molecule or synthesized strand can be denatured using any compound known to dissociate double-stranded nucleic acid molecules, such as any combination of formamide, urea, DMSO, alkali conditions (e.g., NaOH at about 0.01 – 0.3 M, or about 0.05 – 0.1 M; e.g., elevated pH of about 7-12), or low salt or very-low salt conditions (e.g., about less than 0.001 – 0.3 mM cationic conditions), or water.

[0074] In practicing the reagent exchange methods, the target molecule, synthesized strand, polymerase, primer, capture molecule, or any reagent can be removed using fluid flow, washing, or aspiration. The target molecule, primer molecule, synthesized strand, or capture molecule can be operably linked to the solid surface in a manner which withstands flowing, washing, aspirating, and changes in salt, temperature, chemical, enzymatic, or pH conditions. A fresh supply of polymerase, nucleotides,

reagents, primer molecules, splinter molecules, or adaptor molecules, can be added to the immobilized nucleic acid molecules. The polymerase (e.g., donor-labeled) and nucleotides (e.g., and acceptor-labeled) can be added to the immobilized nucleic acid molecules under conditions which are suitable for nucleotide binding or nucleotide incorporation to occur. The fresh polymerase, nucleotides, and reagents, can be the same or different from the old polymerase, nucleotides, or reagents.

[0075] In the following embodiments (e.g., FIG. s 4-10), the “N” can be any nucleotide base, and the “T” can be a universal base such as inosine.

[0076] In one embodiment, a target molecule can be ligated to an immobilized capture molecule using a splinter oligonucleotide (which can hybridize to the target molecule and capture oligonucleotide) and enzymes for ligation or nucleotide polymerization (e.g., T4 ligase and T4 DNA polymerase, respectively). A primer can be annealed to the immobilized target molecule, and a synthesized strand can be produced using a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the synthesized strand, polymerase, and nucleotides. The remaining target molecule can be contacted with fresh reagents to permit re-sequencing the same target molecule. FIG. 4 and FIG. 5 depict using an immobilized target molecule/primer duplex to re-sequence the same target molecule, in a direction away from solid surface, using the reagent exchange methods.

[0077] A two-pass method for re-sequencing the same nucleic acid molecule has been described (Harris, et al., 2008 Science 320:106-109, and supporting online material).

[0078] In another embodiment, a polynucleotide tail (e.g., poly-A, -G, -C, or -T) can be added to a target molecule, for example, using a terminal transferase enzyme (FIG. 5). The tailed target molecule can be ligated to an immobilized capture molecule using a splinter oligonucleotide (which can hybridize to the target molecule and capture oligonucleotide) and enzymes for ligation or nucleotide polymerization (e.g., T4 ligase and T4 DNA polymerase, respectively). A primer can be annealed to the immobilized target molecule, and a synthesized strand can be produced using a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the synthesized strand, polymerase, and nucleotides. The remaining target molecule can be contacted with fresh reagents to permit re-sequencing the same target molecule. FIG. 6 depicts another embodiment using an immobilized, self-primed

target molecule to re-sequence the same target molecule, in a direction away from the solid surface, using the reagent exchange methods.

[0079] FIG. 6 depicts one embodiment using an immobilized target molecule/primer duplex to synthesize an extension product, where the same extension product is re-sequenced in a direction towards the solid surface, using the reagent exchange methods. As shown herein, a target molecule can be ligated to an immobilized hairpin capture molecule, where a portion of the capture molecule can hybridize to the target molecule. The target molecule can be ligated to the hairpin capture molecule using enzymes for ligation or nucleotide polymerization (e.g., T4 ligase and T4 DNA polymerase, respectively). The hairpin adaptor molecule can include a recognition sequence for cleavage (scission) by an endonuclease enzyme. For example, the recognition sequence can be an RNA portion which can be 3-6 nt in length, to form a DNA/RNA hybrid. The RNA portion can be 4 nt in length. The RNA portion can include purines (A and G) in any order. The RNA portion of the RNA/DNA duplex can be a substrate for cleavage by an endoribonuclease (e.g., RNase H). In another example, the recognition sequence can be an AP site (apurinic/aprimidinic) having a THF substrate (tetrahydrofuran) which can be cleaved by an AP endonuclease. In another example, the recognition sequence can include nucleotide analogs (e.g., 8-oxo-7,8-dihydroguanine, 8-oxoguanine, or 8-hydroxyguanine) that can be cleaved by DNA glycosylase OGG1. In yet another example, the recognition sequence can include any sequence that can be cleaved by a nicking enzyme. After scission, a primer can be annealed to the target molecule, and a synthesized strand can be produced using a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the synthesized strand, polymerase, and nucleotides. The remaining target molecule can be contacted with fresh reagents to permit re-sequencing the same target molecule.

[0080] FIG. 7 depicts another embodiment using an immobilized target molecule/primer duplex to synthesize an extension product, where the same extension product is re-sequenced in a direction towards the solid surface, using the reagent exchange methods. As shown herein, the 5' end of a target molecule can be ligated to an adaptor molecule using T4 ligase. The adaptor molecule can be annealed with a primer having a blocked 3' end. The target molecule can be reacted with terminal transferase to add a poly-nucleotide tail (e.g., poly-A, -G, -C, or -T). The tailed target

molecule can be captured by an immobilized oligonucleotide. The immobilized oligonucleotide can be used to produce a synthesized strand, using a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the target strand, polymerase, and nucleotides. A primer can be annealed to the remaining synthesized strand. A newly synthesized strand can be produced using a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the newly synthesized strand, polymerase, and nucleotides. The remaining synthesized strand can be contacted with fresh reagents to permit re-sequencing the same synthesized strand.

[0081] In yet another embodiment, the target molecule can be reacted with terminal transferase to add a poly-nucleotide tail (e.g., poly-A, -G, -C, or -T) (TdT in FIG. 8). The tailed target molecule can be captured by an immobilized capture oligonucleotide (FIG. 8). The immobilized capture oligonucleotide can be used to generate a synthesized strand, using a polymerase and nucleotides (FIG. 8). The 3' end of the synthesized strand can be ligated to an adaptor molecule. Physical, chemical, or enzymatic conditions can be used to remove the target molecule, polymerase, and nucleotides. The 3' end of the remaining synthesized strand can be annealed to a primer. A newly synthesized strand can be generated with a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the newly synthesized strand, polymerase, and nucleotides. The remaining synthesized strand can be contacted with fresh reagents to permit re-sequencing the same synthesized strand. FIG. 8 depicts re-sequencing the same synthesized strand, in a direction towards the solid surface.

[0082] FIG. 9 depicts one embodiment using an immobilized circular target nucleic acid molecule and a primer for rolling circle replication to re-sequence the same target molecule multiple times. As shown herein, the target molecule can be reacted with terminal transferase to add a poly-nucleotide tail (e.g., poly-A, -G, -C, or -T). The tailed target molecule can be captured by an immobilized capture oligonucleotide. The immobilized capture oligonucleotide can be used to generate a synthesized strand, using a polymerase and nucleotides. The 3' end of the synthesized strand can be ligated to an adaptor molecule. Physical, chemical, or enzymatic conditions can be used to remove the target molecule, polymerase, and nucleotides. The 3' end of the remaining synthesized strand can be annealed to a primer. A newly synthesized

strand can be generated with a polymerase and nucleotides. Physical, chemical, or enzymatic conditions can be used to remove the newly synthesized strand, polymerase, and nucleotides. The remaining synthesized strand can be contacted with fresh reagents to permit re-sequencing the same synthesized strand.

[0083] FIG. 10 depicts an embodiment using an immobilized double-stranded target nucleic acid molecule, which is ligated at both ends with adaptors, for rolling circle replication to re-sequence the same target molecule multiple times. The circularized target molecule can be captured by an immobilized oligonucleotide. The 3' end of the capture oligonucleotide can be used to generate a synthesized strand using a polymerase and nucleotides, in a rolling circle replication mode. A strand-displacement DNA polymerase can be used for the rolling circle replication.

Kits

[0084] Provided herein are kits for conducting the nucleotide binding reactions or the nucleotide incorporation reactions described herein. The kits can include, in one or more containers, the components of nucleotide binding or nucleotide incorporation disclosed herein, including: the solid surfaces, energy transfer moieties, reporter moieties, nanoparticles, polymerases, nucleotide polyphosphate molecules, target nucleic acid molecules (e.g., a control test target molecules), primers, or oligonucleotides.

[0085] In the kits, the solid surfaces, energy transfer moieties, reporter moieties, nanoparticles, polymerases, nucleotide polyphosphate molecules, target nucleic acid molecules, primers, or oligonucleotides can be attached to each other in any combination, or be unattached. The kits can include positive or negative control samples.

[0086] Additional components can be included in the kit, such as buffers and reagents. For example, the buffers can include Tris, Tricine, HEPES, or MOPS, or chelating agents such as EDTA or EGTA. In another example, the reagents can include monovalent ions, such as KCl, K-acetate, NH₄-acetate, K-glutamate, NH₄Cl, or ammonium sulfate. In yet another example, the reagents can include divalent ions, such as Ca²⁺, CaCl₂, Mg²⁺, MgCl₂, Mg-acetate, Mn²⁺, MnCl₂, and the like. The kits can include the components in pre-measured unit amounts. The kits can include instructions for performing the nucleotide binding reactions or the nucleotide

incorporation reactions. Where the kit is intended for diagnostic applications, the kits may further include a label indicating regulatory approval for the diagnostic application.

EXAMPLES

Example 1:

Reagent Exchange Methods

Oligonucleotides

Target molecule:

5'TTTTGA TTTTTTTTTTTT CCCCCCCCCC TTTTTTTTTTTT
CCCCCCCCCCCC g ACA Cgg Agg TTC TAT CAT CgT CAT CgT CAT CgT CAT
Cg -amine-3'

Primer molecule A for cycle 1:

5' TGA TAG AAC CTC CGT GTC 3'

Primer molecule B for cycle 2:

5' TGA TAG AAC CTC YGT GTC 3' (Y = amino modifier C6, C is base, labeled with AF647)

1X TBST/BSA Wash buffer:

[0087] 50 mM Tris pH 7.5; 50 mM NaCl; 0.05% Tween-20; 0.5% BSA.

1X Polymerase binding buffer:

[0088] 50 mM MOPS pH 6.8; 100 mM NaCl; 0.1 % BSA.

Pre-Extension mix G.O./Cat OSS:

[0089] 50 mM MOPS pH 7.2 w/KOH; 50 mM potassium acetate (KOAc) pH 7.0; 2 mM Trolox (dissolved 24 mM MOPS pH 6.8; stored at -20 °C); 0.2% cyclooctatetraene; 100 U/mL glucose oxidase; 10 U/μL Catalase; 0.4% glucose.

Extension mix G.O./Cat OSS:

[0090] 50 mM MOPS pH 7.2 w/KOH; 50 mM KOAc pH 7.0; 2 mM Trolox (dissolved 24 mM MOPS pH 6.8; stored at -20 °C); 0.2 % cyclooctatetraene; 100 U/mL glucose oxidase; 10 U/ μ L Catalase; 0.4% glucose; 0.6 mM MnCl_2 ; 100 nM AF647-dG6P; 100 nM AF676-dA6P.

Covalent-DNA Immobilization and Chip Preparation:

[0091] Coverslips from MicroSurfaces, Inc. are prepared as follows. The lane is injected with 300 pM 434 target molecules primed with 315 primer, is dissolved in 500 mM borate pH 8.2 and is incubated for approximately 5 minutes. The reaction is terminated with 0.1 mL wash (500 mM Borate, pH 8.2). NHS deactivation is conducted using Deactivation buffer supplied by MicroSurfaces, Inc., by injecting 0.08 mL/lane and is incubated for more than 5 minutes. The chip is washed with 1X TBST/BSA (1 mL/lane). The chip is mounted on the scope.

Cycle 1 Nucleotide Incorporation Reaction:

[0092] Polymerase binding buffer wash (0.3 mL/lane) is injected. 2-5 nM of the polymerase conjugate (Cy3-SA-Phi29 mutant) is injected and is incubated until desired density is reached (~ 900 spots/FOV).

[0093] Polymerase binding buffer wash (0.2 mL/lane) is injected. Pre-extension mix (without nucleotides) ~3-5 min (0.1 mL/lane) is injected. 1X extension mix with nucleotides (0.1 mL/lane) is injected. For cycle 1, AF647-dG6P and AF676-dA6P terminal phosphate labeled nucleotides are used.

Removal of Polymerase and Synthesized Strand:

[0094] The polymerase used in cycle 1 is removed using 6.3 M guanidine isothiocyanate, 160 mM Tris pH 9.7, and 2.6 mM EGTA. The synthesized strand is removed using 25% Formamide, 50 mM NaOH.

Cycle 2 Exchanged Polymerase and Primer:

[0095] For cycle 2, 500 nM of fresh, AF647- labeled 336 primer is added in 1X TBST/BSA, and is incubated for 5 minutes.

[0096] Polymerase binding buffer wash (0.3 mL/lane) is injected. Approximately 2-5 nM of the polymerase conjugate (Cy3-SA-Phi29 mutant) is injected.

[0097] Polymerase binding buffer wash (0.2 mL/lane) is injected. Pre-extension mix (without nucleotides) ~3-5 min (0.1 mL/lane) is injected. 1X extension mix with nucleotides (0.1 mL/lane) is injected. For cycle 2, AF676-dG6P and AF700-dA6P terminal phosphate labeled nucleotides are used.

EXAMPLE 2

[0098] Synthesis is performed using different dye pairings, influencing the probability of deletion and the branch ratio for particular nucleotides. Dye set 1 includes labeled nucleotides: dC-P2-Dy634, dG-P2-AF647, dA-P2-AF676, and dT-P2-AF700. Dye set 2 includes labeled nucleotides dT-P2-Dy634, dG-P2-AF647, dA-P2-AF676, and dC-P2-AF700. As illustrated in FIG. 11 and in Table 1 and Table 2 below, the selection of dye pairings significantly changes the branching ratio (BR) and probability of deletion (Pd) for the T and C nucleotides.

[0099] TABLE 1. Branch Ratio and Deletion Rate for Dye Set 1

Dye Set 1	A-P2- 676	G- P2- 647	C- P2- 634	T- P2- 700
Event Duration	9.2	10	12	6.8
Inter Event	12.1	12.8	11.9	12.7
Branch Ratio	0.78	0.73	0.6	0.77
Deletion Rate	0.14	0.13	0.16	0.28

[00100] TABLE 2. Branch Ratio and Deletion Rate for Dye Set 2

Dye Set 2	A-C6- 676	G-C6- 647	C-C6- 634	T-C6- 700
Event Duration	9.5	8.8	7.4	7.6
Inter Event	11.5	11.9	12.4	16.1
Branch Ratio	0.81	0.71	0.8	0.46
Deletion Rate	0.16	0.33	0.21	0.23

[00101] Resequencing using different dye sets can compensate for systematic error, providing for improved accuracy.

[00102] EXAMPLE 3

[00103] Synthesis is performed at different pH, which influences event duration and incorporation rate. Synthesis is performed at pH 7.4 or pH 7.8 for 125 nM dye-nucleotide solution or 250 nM dye-nucleotide solution. As illustrated in FIG. 12, FIG. 13, and FIG. 14, an increase in pH increases the incorporation rate and decreases the event duration. Adjusting sequencing conditions during or between cycles can adjust incorporation rates and event duration, which can influence detection of the incorporation events for different nucleotides. By resequencing under different conditions, systematic errors associated with detection can be reduced.

[00104] EXAMPLE 4

[00105] Synthesis is performed at different pH and branch ratio is observed. The pH is selected from pH 7.5, pH 8, and pH 8.5, illustrated in FIG. 15 from left to right in association with each nucleotide. As illustrated in FIG. 15, increasing pH increases branch ratio for each of the nucleotides. By adjusting conditions in an automated manner, a balance can be obtained between branch ratio and event duration. Using a low pH provides a low branch ratio, but longer durations, providing more accurate color transitions with better detectability. At high pH, the branch ratio is higher, but the event durations are short, which is useful in counting bases. Combinations of cycles at different conditions can provide different data that in combination leads to a reduction in detection errors and other systematic or random errors when analyzed.

[00106] In an embodiment, a sequencing system includes a sequencing reaction chamber for receiving an immobilized nucleic acid target, a sequencing analytics component, and a sequencing attribute controller in communication with the sequencing analytics component and in communication with the sequencing reaction chamber. The sequencing attribute controller is to control a sequencing attribute of the sequencing reaction chamber in response to output of the sequencing analytics component.

[00107] In an example of the embodiment, the sequencing system further includes a detection component interfaced to the sequencing reaction chamber and in communication with the sequencing analytics component. The detection component is to detect nucleotide incorporation on the nucleic acid target and is to provide a signal to the sequencing analytics component indicative of the nucleotide incorporation. In an example, the nucleotide incorporation is characterized by a

fluorescent signal and the detection component includes an optical detection component. The optical detection component can include a CMOS imaging component. The optical detection component can include a charge coupled device (CCD) component. In an additional example, the sequencing attribute controller is in communication with the detection component. The sequencing attribute controller is to control a hardware attribute of the detection component in response to the output from the sequencing analytics component. For example, the hardware attribute includes wavelength.

[00108] In a further example, the output of the sequencing analytics component is associated with branch ratio. In an additional example, the output of the sequencing analytics component is associated with deletion probability.

[00109] In another example, the sequencing attribute includes pH. In an additional example, the sequencing attribute includes alternate dye nucleotide pairings. In a further example, the sequencing attribute includes an alternate polymerase. For example, the alternate polymerase includes a select energy transfer donor. In another example, the sequencing attribute includes a nucleotide analog.

[00110] In a further embodiment, a sequencing system includes a sequencing reaction chamber for receiving an immobilized nucleic acid target and a fluorescent dye labeled nucleotide and includes a detection component interfaced to the sequencing reaction chamber. The detection component is to detect incorporation of the fluorescent dye labeled nucleotide on the immobilized nucleic acid target. The sequencing system further includes a sequencing analytics component in communication with the detection component. The sequencing component is to receive a signal from the detection component associated with detecting incorporation of the fluorescent dye labeled nucleotide. In addition, the sequencing system includes a sequencing attribute controller in communication with the sequencing analytics component and in communication with the sequencing reaction chamber. The sequencing attribute controller is to control a sequencing attribute of the sequencing reaction chamber or a hardware attribute of the detection component in response to output of the sequencing analytics component.

[00111] In an additional embodiment, a method of sequencing a nucleic acid target includes performing a first synthesis with a first sequencing attribute in a sequencing reaction chamber, determining with a sequencing analytics component a performance

parameter associated with the first synthesis, and performing a second synthesis with a second sequencing attribute selected by a sequencing attribute controller based on the performance parameter.

[00112] In an example, the method further includes detecting with a detecting component a nucleotide incorporation event associated with the first synthesis; and providing a signal indicative of detecting to the sequencing analytics component. For example, the method can further include adjusting with the sequencing attribute controller a hardware attribute associated with the detecting component.

[00113] In another example, the first and second sequencing attributes include pH. In an additional example, the first and second sequencing attributes are associated with different dye nucleotide pairings. In a further example, the first and second sequencing attributes are associated with different polymerases.

[00114] Note that not all of the activities described above in the general description or the examples are required, that a portion of a specific activity may not be required, and that one or more further activities may be performed in addition to those described. Still further, the orders in which activities are listed are not necessarily the order in which they are performed.

[00115] In the foregoing specification, the concepts have been described with reference to specific embodiments. However, one of ordinary skill in the art appreciates that various modifications and changes can be made without departing from the scope of the invention as set forth in the claims below. Accordingly, the specification and figures are to be regarded in an illustrative rather than a restrictive sense, and all such modifications are intended to be included within the scope of invention.

[00116] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having” or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, method, article, or apparatus that comprises a list of features is not necessarily limited only to those features but may include other features not expressly listed or inherent to such process, method, article, or apparatus. Further, unless expressly stated to the contrary, “or” refers to an inclusive-or and not to an exclusive-or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

[00117] Also, the use of “a” or “an” are employed to describe elements and components described herein. This is done merely for convenience and to give a general sense of the scope of the invention. This description should be read to include one or at least one and the singular also includes the plural unless it is obvious that it is meant otherwise.

[00118] Benefits, other advantages, and solutions to problems have been described above with regard to specific embodiments. However, the benefits, advantages, solutions to problems, and any feature(s) that may cause any benefit, advantage, or solution to occur or become more pronounced are not to be construed as a critical, required, or essential feature of any or all the claims.

[00119] After reading the specification, skilled artisans will appreciate that certain features are, for clarity, described herein in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features that are, for brevity, described in the context of a single embodiment, may also be provided separately or in any subcombination. Further, references to values stated in ranges include each and every value within that range.

CLAIMS

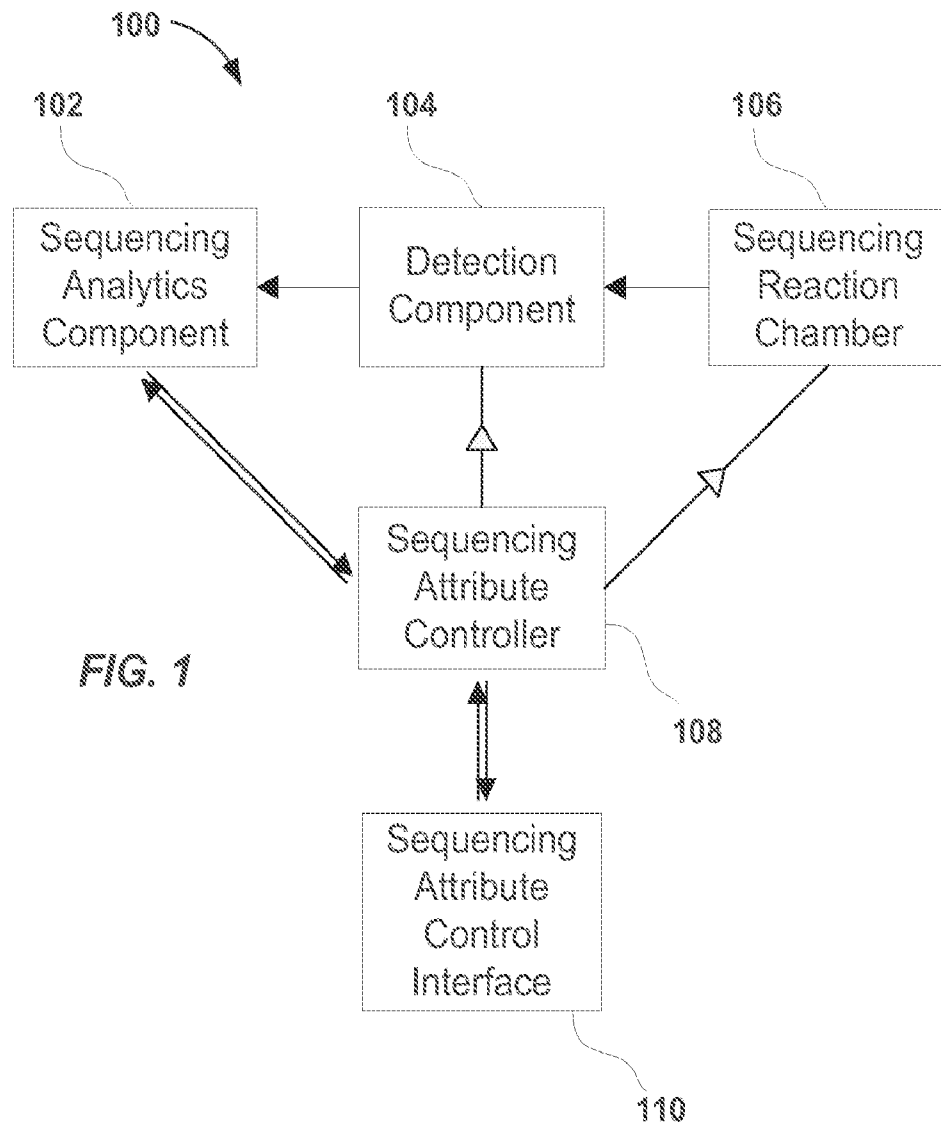
1. A sequencing system comprising:
 - a sequencing reaction chamber for receiving an immobilized nucleic acid target;
 - a sequencing analytics component; and
 - a sequencing attribute controller in communication with the sequencing analytics component and in communication with the sequencing reaction chamber;wherein the sequencing attribute controller is to control a sequencing attribute of the sequencing reaction chamber in response to output of the sequencing analytics component.
2. The sequencing system of claim 1, further comprising a detection component interfaced to the sequencing reaction chamber and in communication with the sequencing analytics component, the detection component to detect nucleotide incorporation on the nucleic acid target and to provide a signal to the sequencing analytics component indicative of the nucleotide incorporation.
3. The sequencing system of claim 2, wherein the nucleotide incorporation is characterized by a fluorescent signal, the detection component including an optical detection component.
4. The sequencing system of claim 3, wherein the optical detection component includes a CMOS imaging component.
5. The sequencing system of claim 3, wherein the optical detection component includes a charge coupled device (CCD) component.
6. The sequencing system of claim 2, wherein the sequencing attribute controller is in communication with the detection component, the sequencing attribute controller to

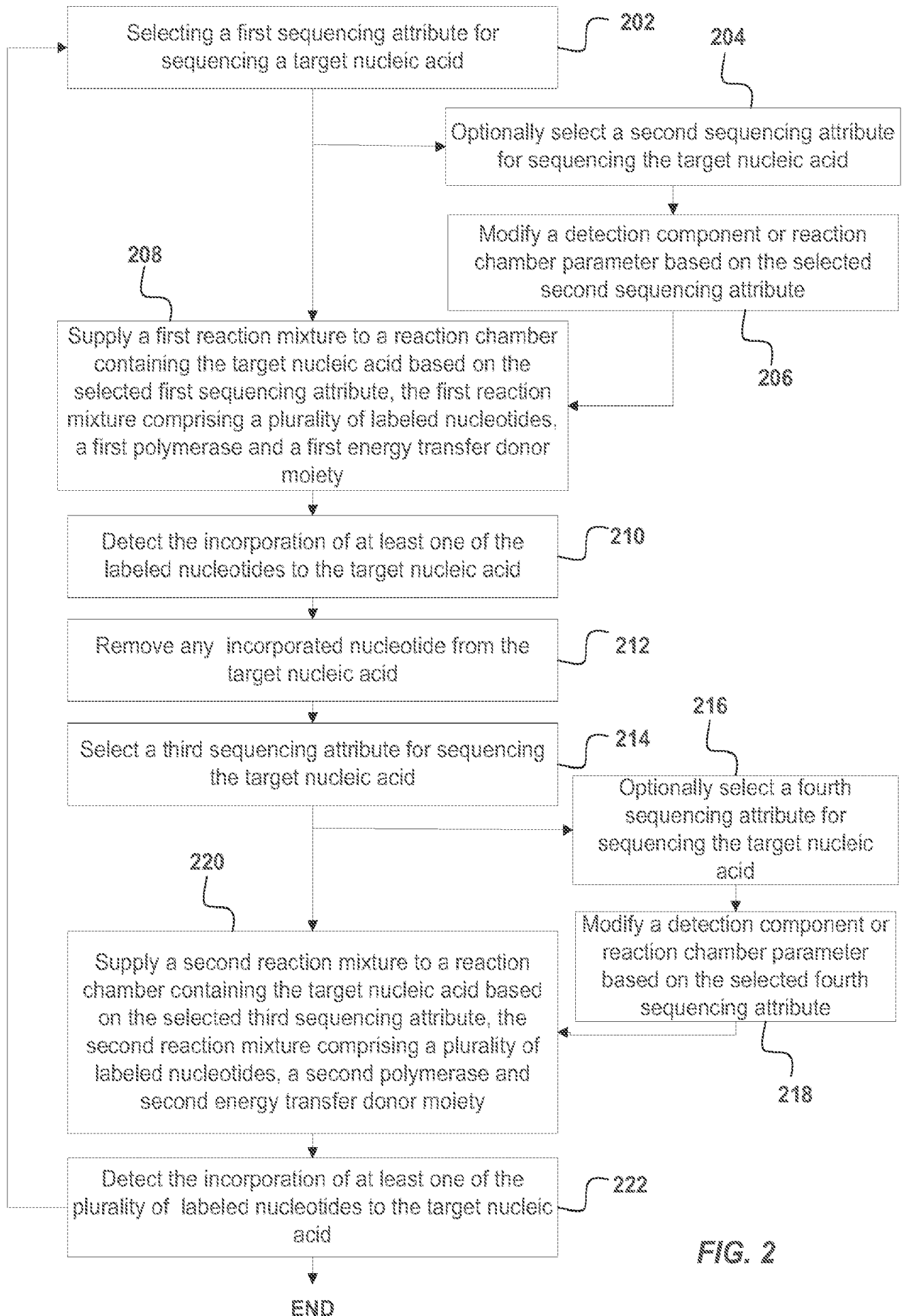
control a hardware attribute of the detection component in response to the output from the sequencing analytics component.

7. The sequencing system of claim 6, wherein the hardware attribute includes wavelength.
8. The sequencing system of any one of claims 1-7, wherein the output of the sequencing analytics component is associated with branch ratio.
9. The sequencing system of any one of claims 1-7, wherein the output of the sequencing analytics component is associated with deletion probability.
10. The sequencing system of any one of claims 1-7, wherein the sequencing attribute includes pH.
11. The sequencing system of any one of claims 1-7, wherein the sequencing attribute includes alternate dye nucleotide pairings.
12. The sequencing system of any one of claims 1-7, wherein the sequencing attribute includes an alternate polymerase.
13. The sequencing system of claim 12, wherein the alternate polymerase includes a select energy transfer donor.
14. The sequencing system of any one of claims 1-7, wherein the sequencing attribute includes a nucleotide analog.
15. A sequencing system comprising:
 - a sequencing reaction chamber for receiving an immobilized nucleic acid target and a fluorescent dye labeled nucleotide;
 - a detection component interfaced to the sequencing reaction chamber, the detection component to detect incorporation of the fluorescent dye labeled nucleotide on the immobilized nucleic acid target;

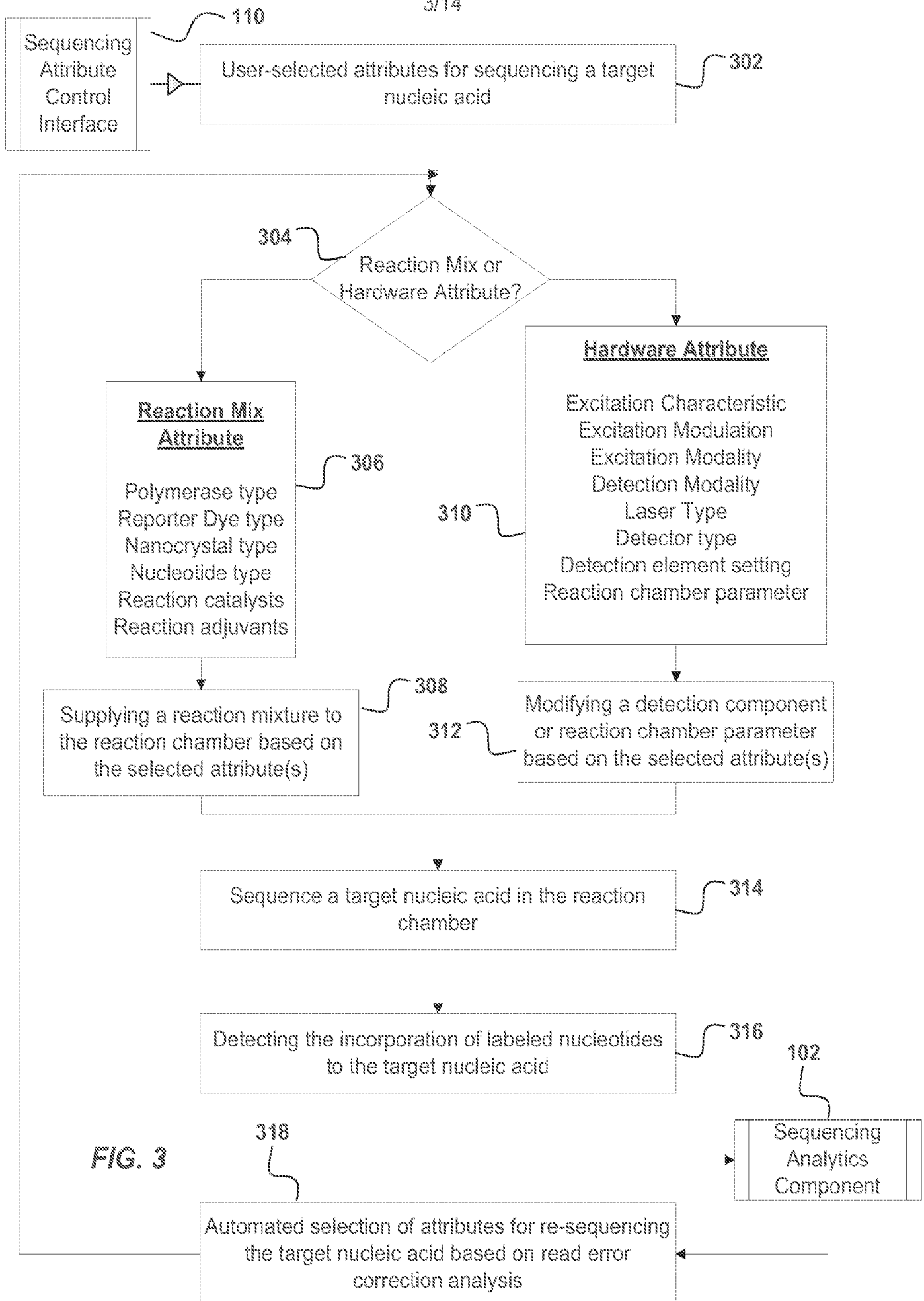
- a sequencing analytics component in communication with the detection component, the sequencing component to receive a signal from the detection component associated with detecting incorporation of the fluorescent dye labeled nucleotide; and
- a sequencing attribute controller in communication with the sequencing analytics component and in communication with the sequencing reaction chamber;
- wherein the sequencing attribute controller is to control a sequencing attribute of the sequencing reaction chamber or a hardware attribute of the detection component in response to output of the sequencing analytics component.
16. A method of sequencing a nucleic acid target, the method comprising:
- performing a first synthesis with a first sequencing attribute in a sequencing reaction chamber;
- determining with a sequencing analytics component a performance parameter associated with the first synthesis; and
- performing a second synthesis with a second sequencing attribute selected by a sequencing attribute controller based on the performance parameter.
17. The method of claim 16, further comprising detecting with a detecting component a nucleotide incorporation event associated with the first synthesis; and providing a signal indicative of detecting to the sequencing analytics component.
18. The method of claim 17, further comprising adjusting with the sequencing attribute controller a hardware attribute associated with the detecting component.
19. The method of 16, wherein the first and second sequencing attributes include pH.
20. The method of 16, wherein the first and second sequencing attributes are associated with different dye nucleotide pairings.

21. The method of claim 16, wherein the first and second sequencing attributes are associated with different polymerases.

**FIG. 1**

**FIG. 2**

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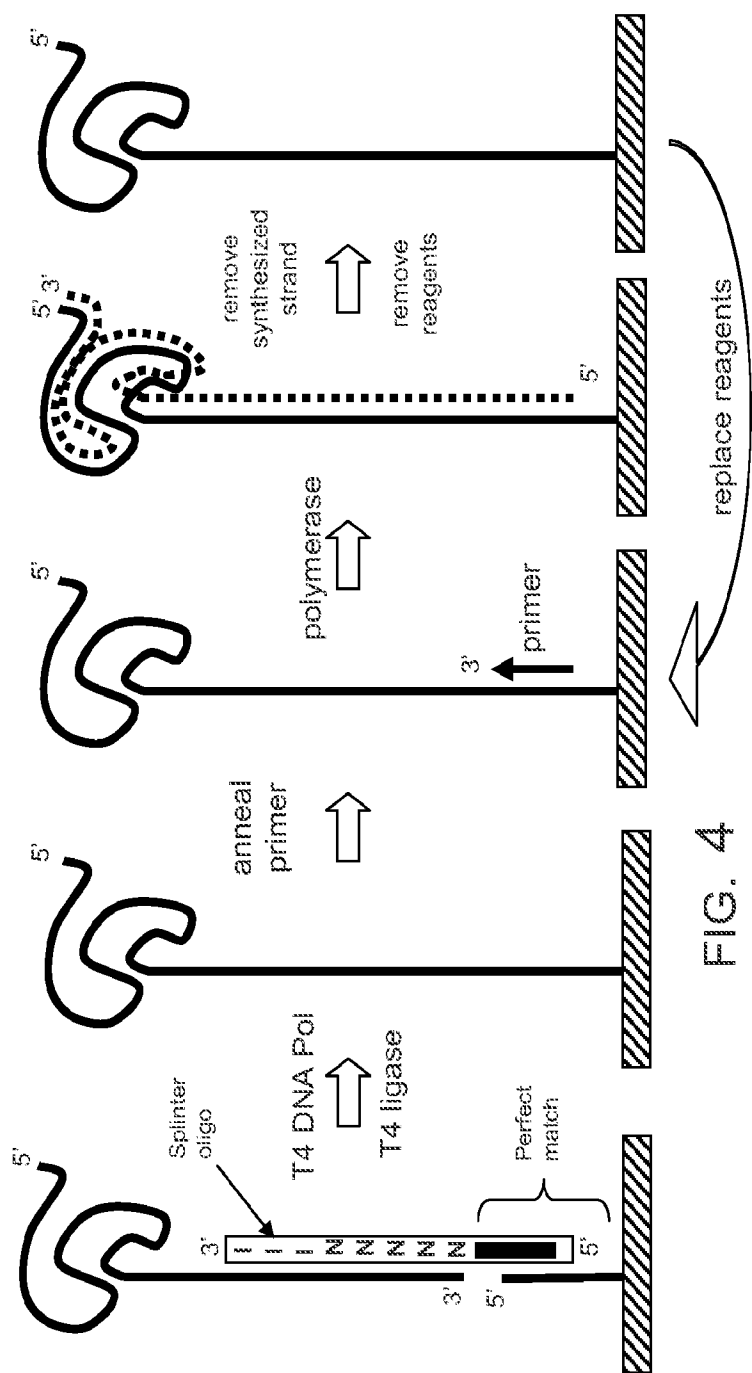


FIG. 4

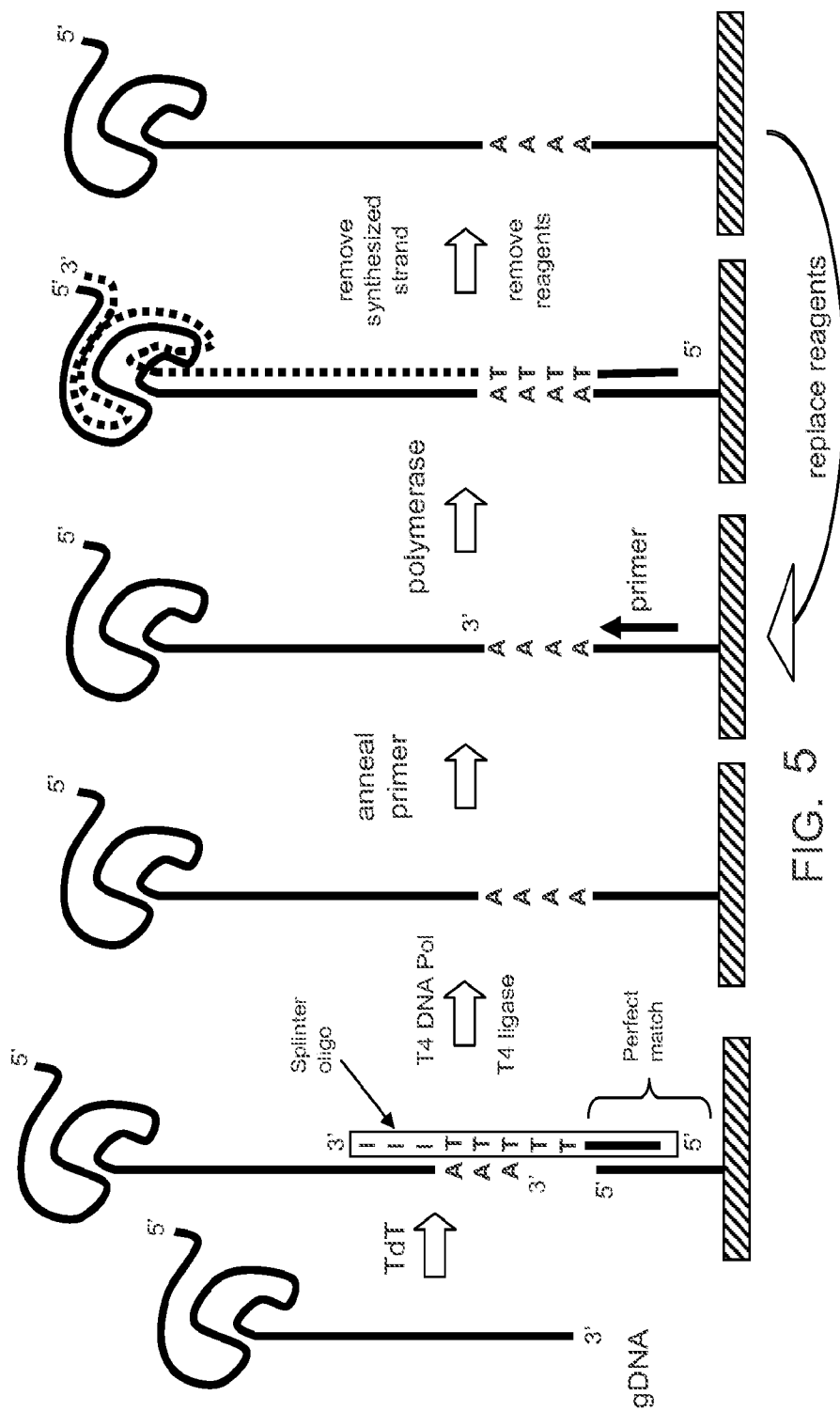


FIG. 5

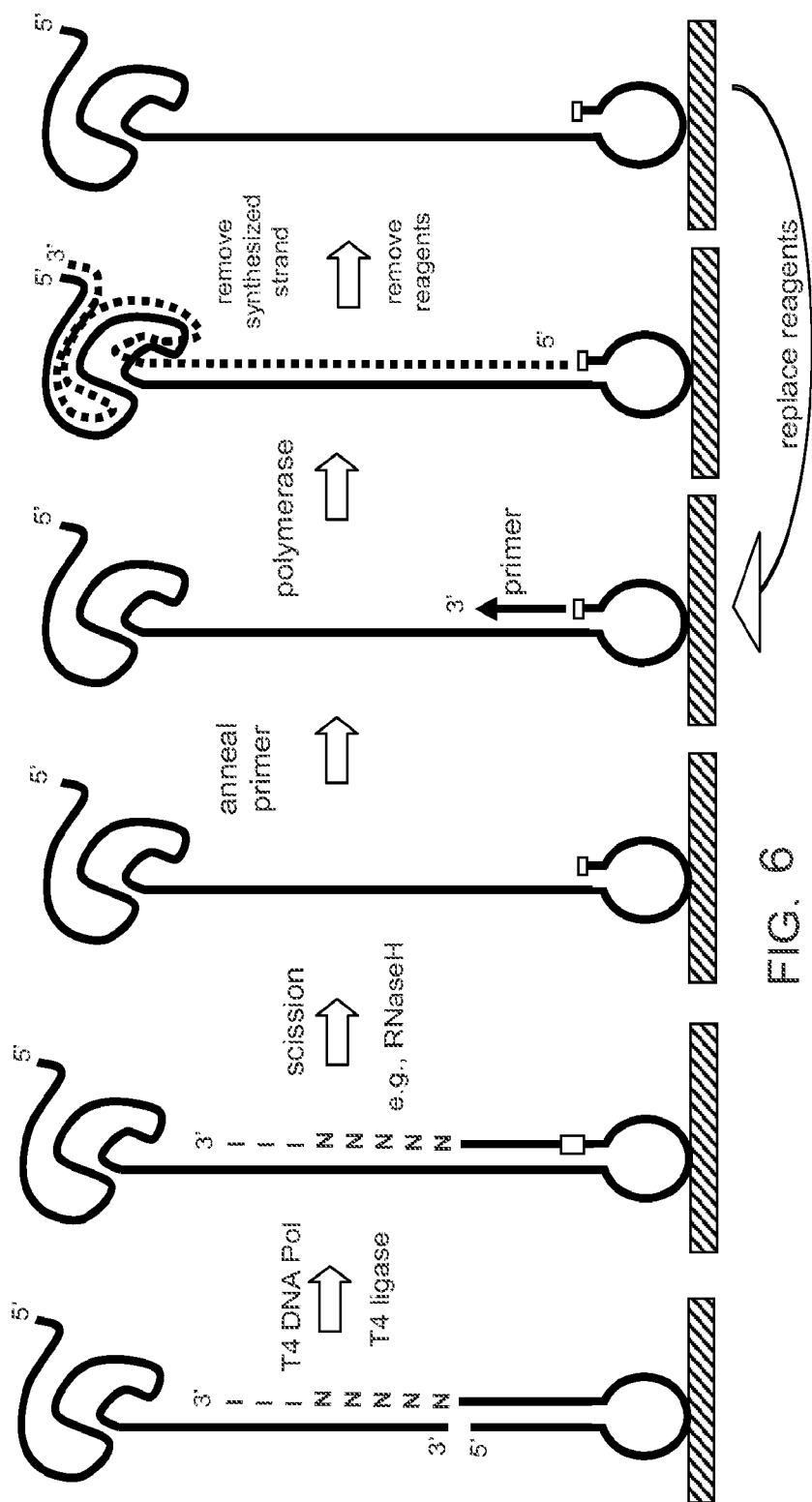


FIG. 6

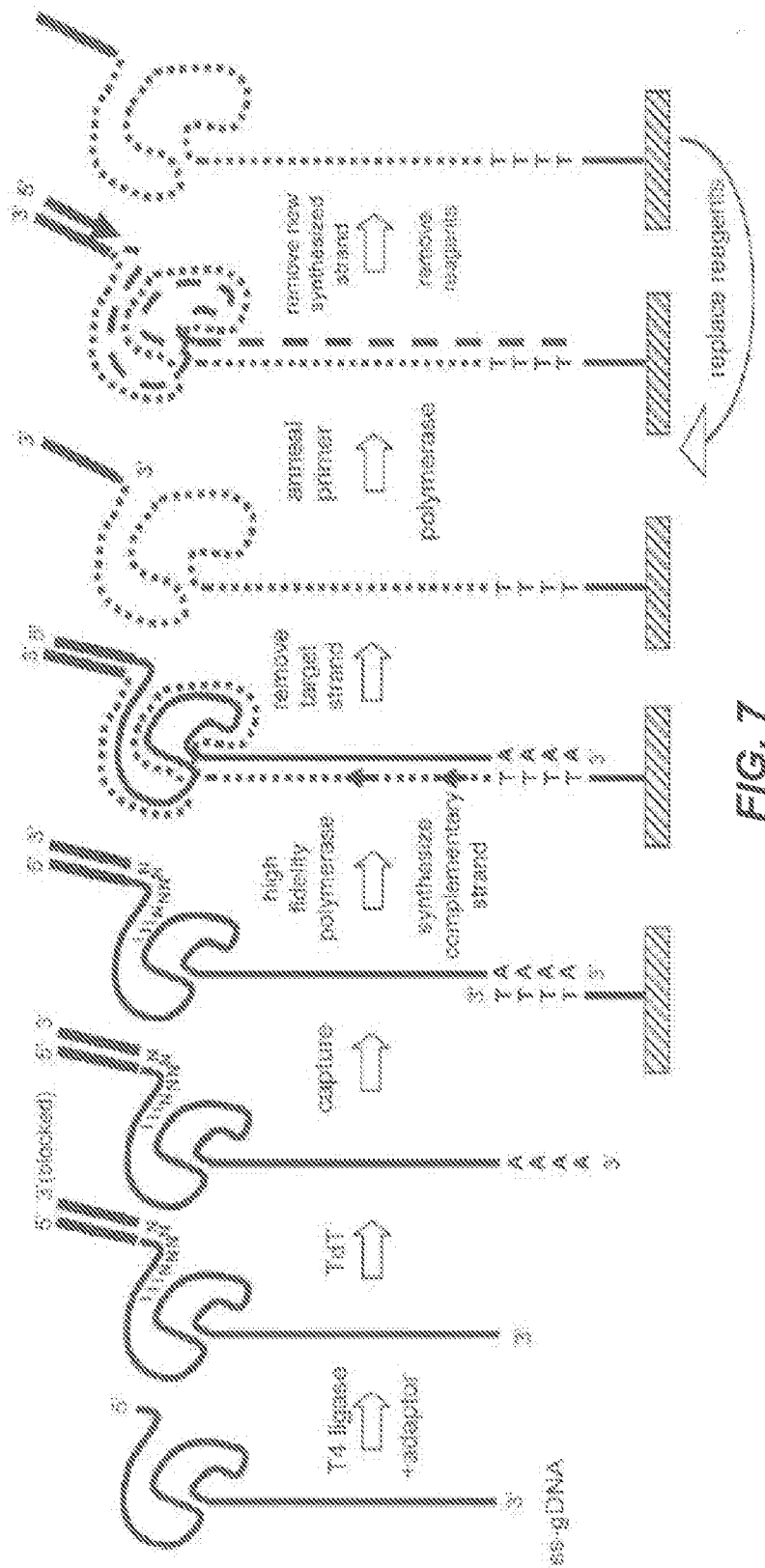


FIG. 7

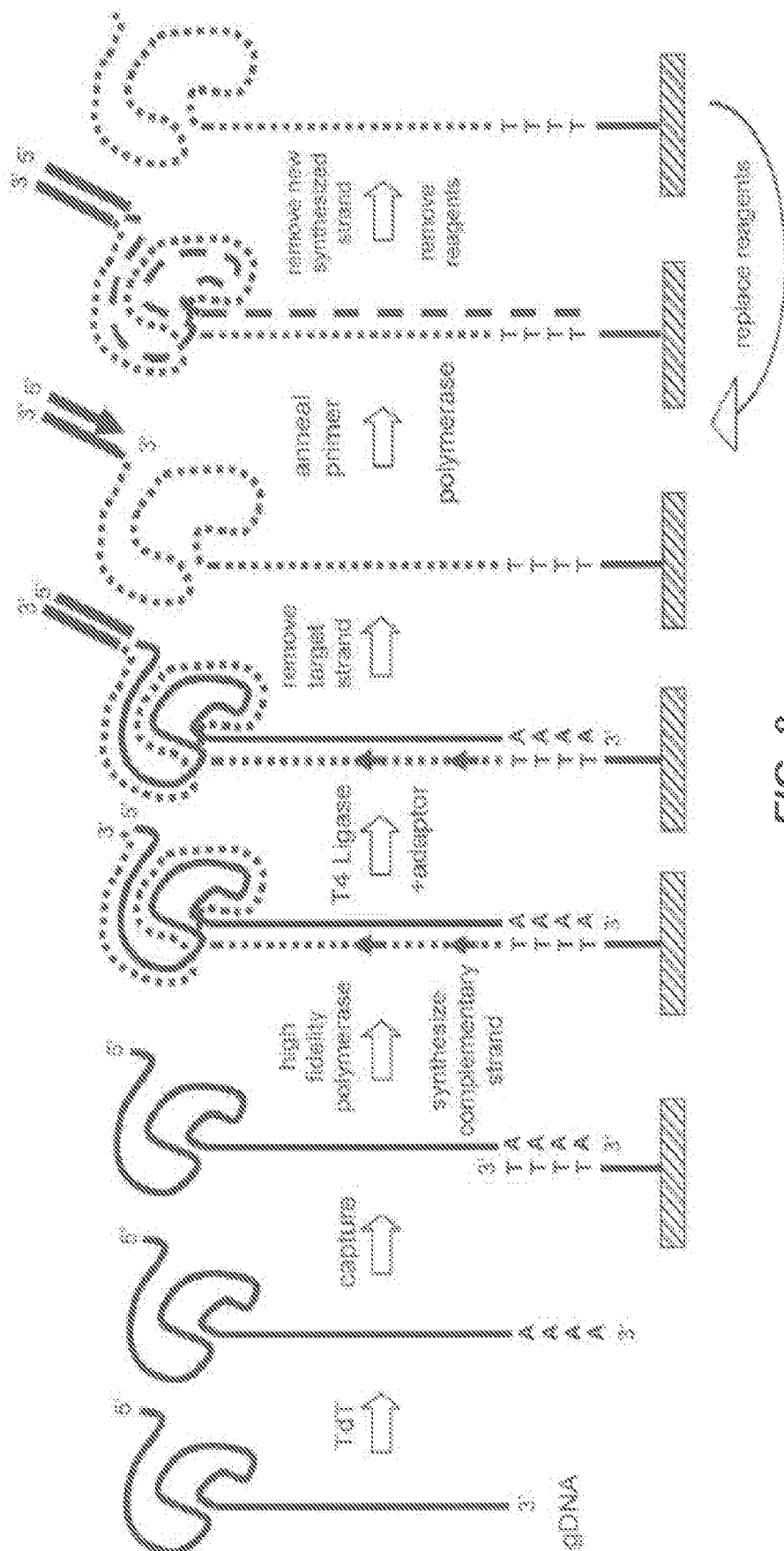


FIG. 8

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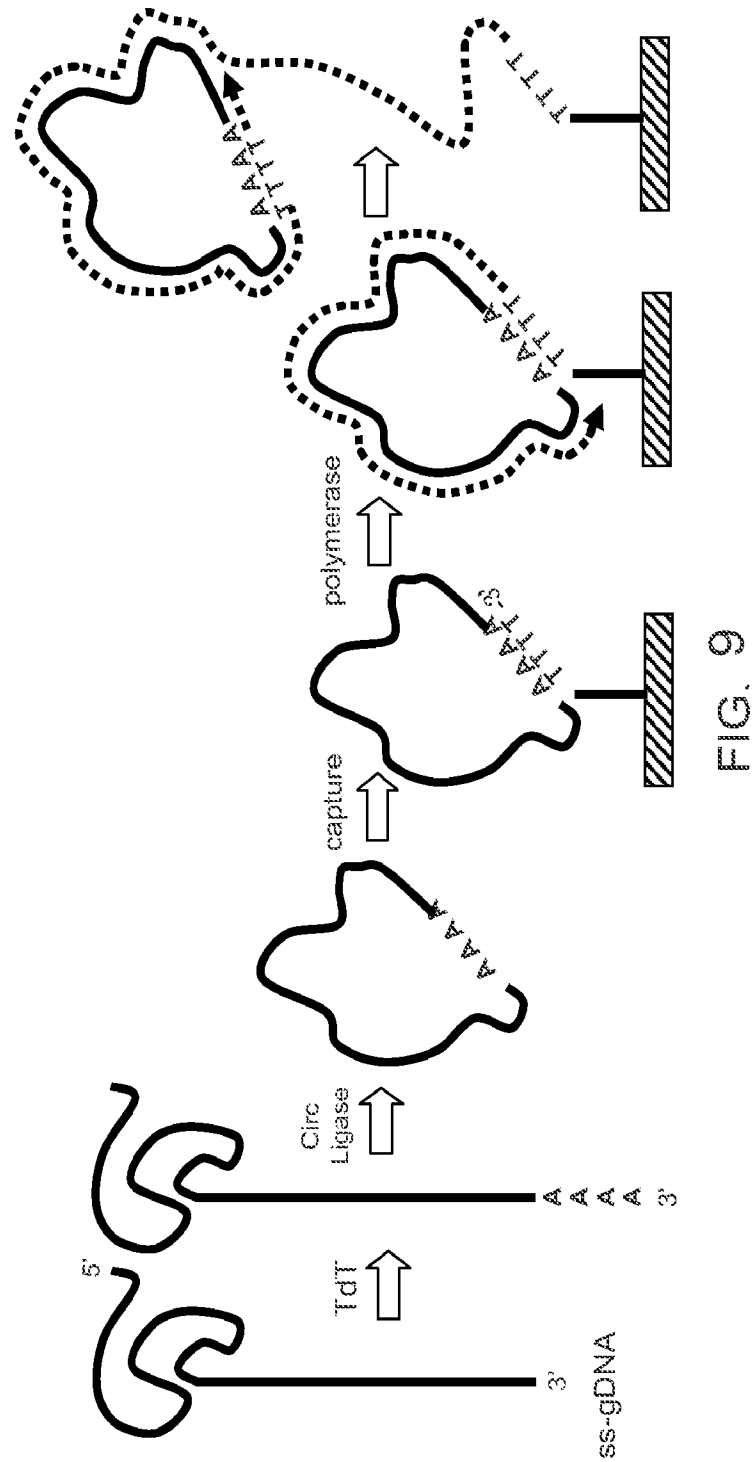


FIG. 9

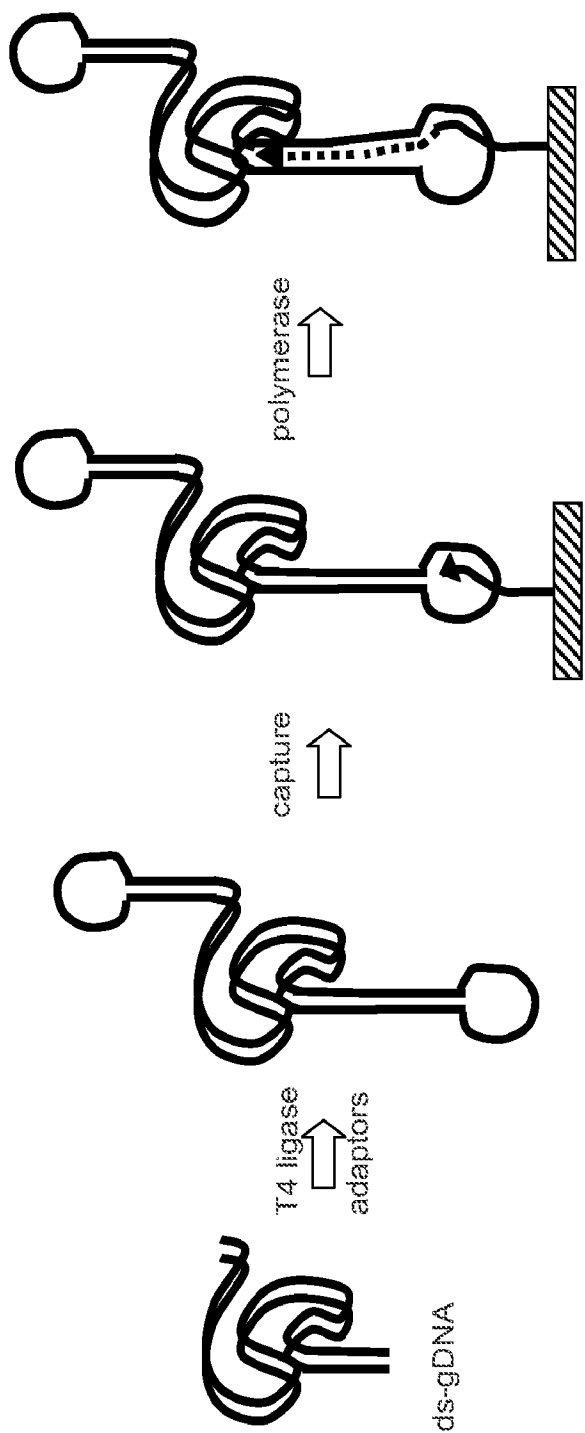


FIG. 10

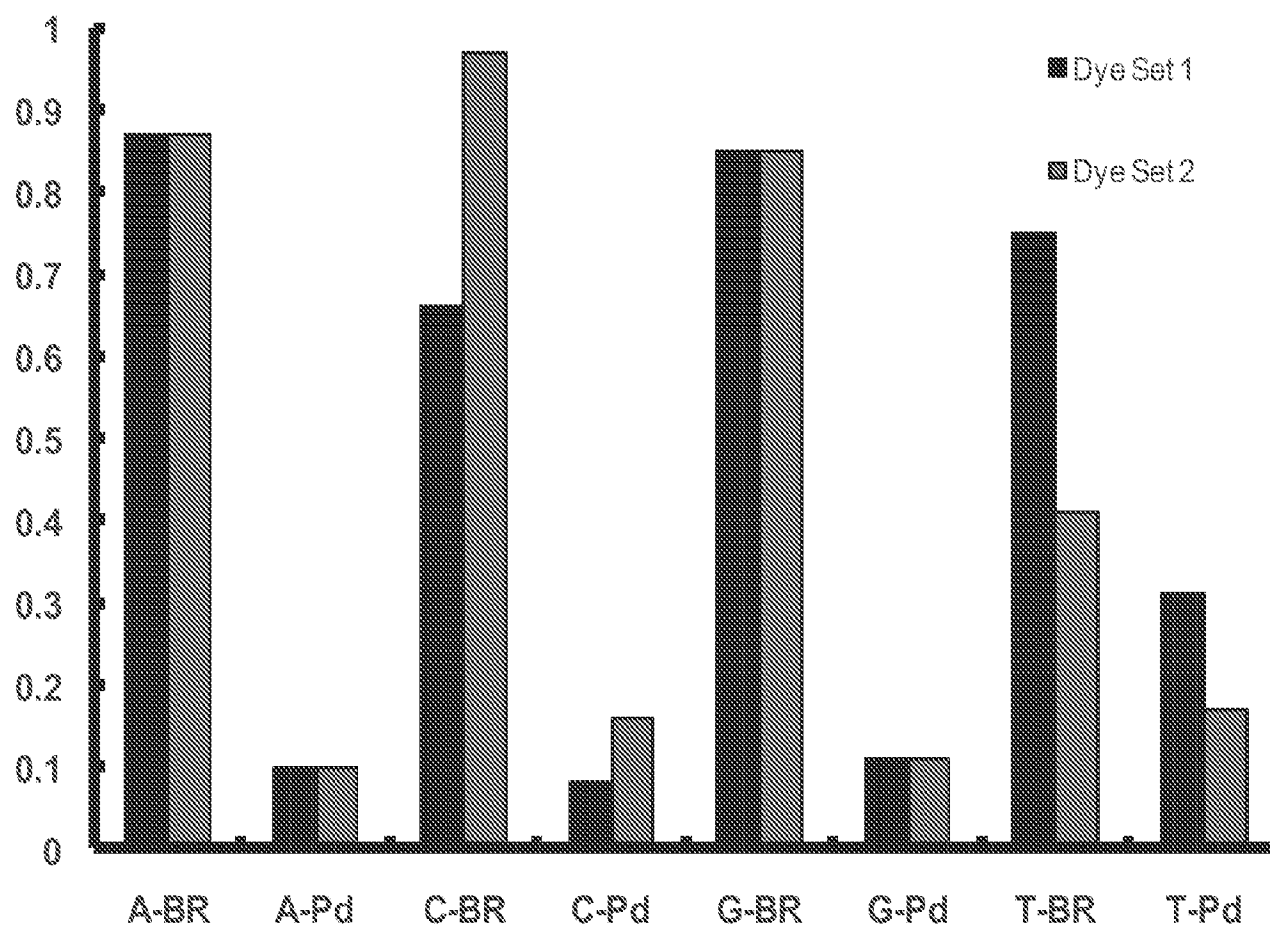


FIG. 11

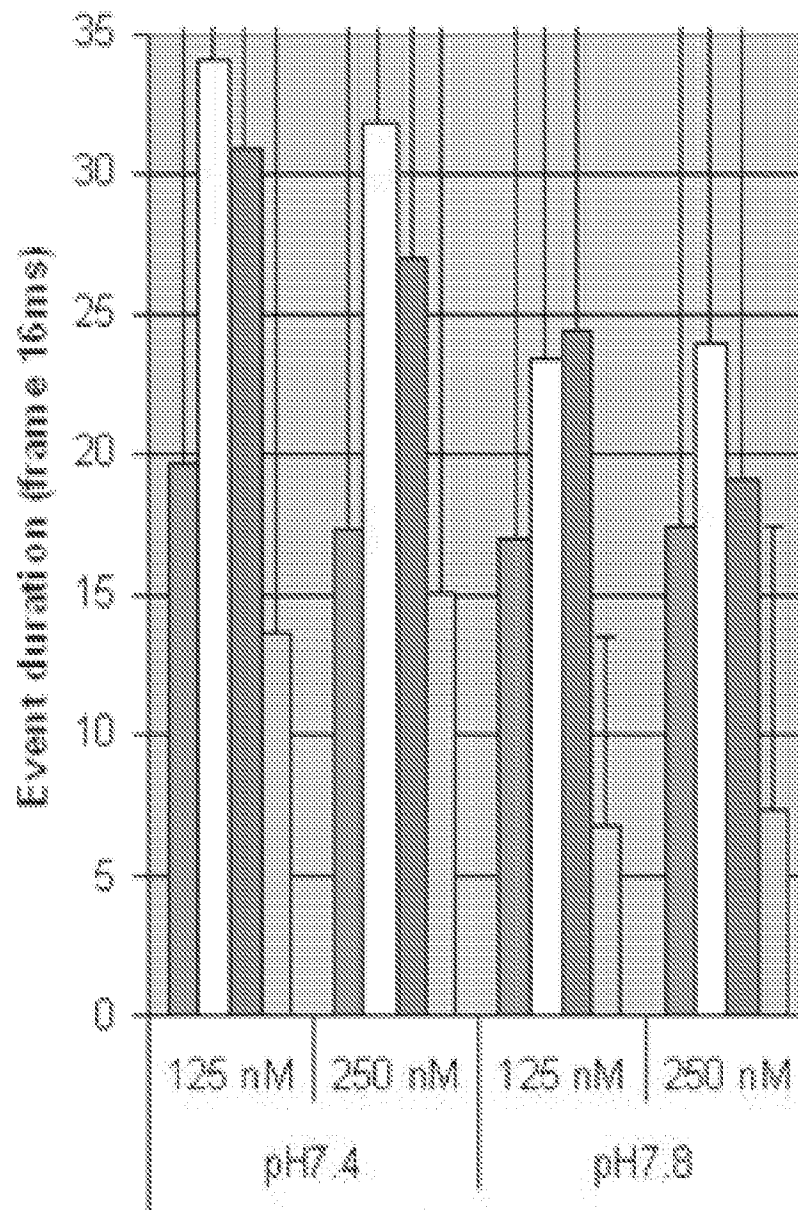
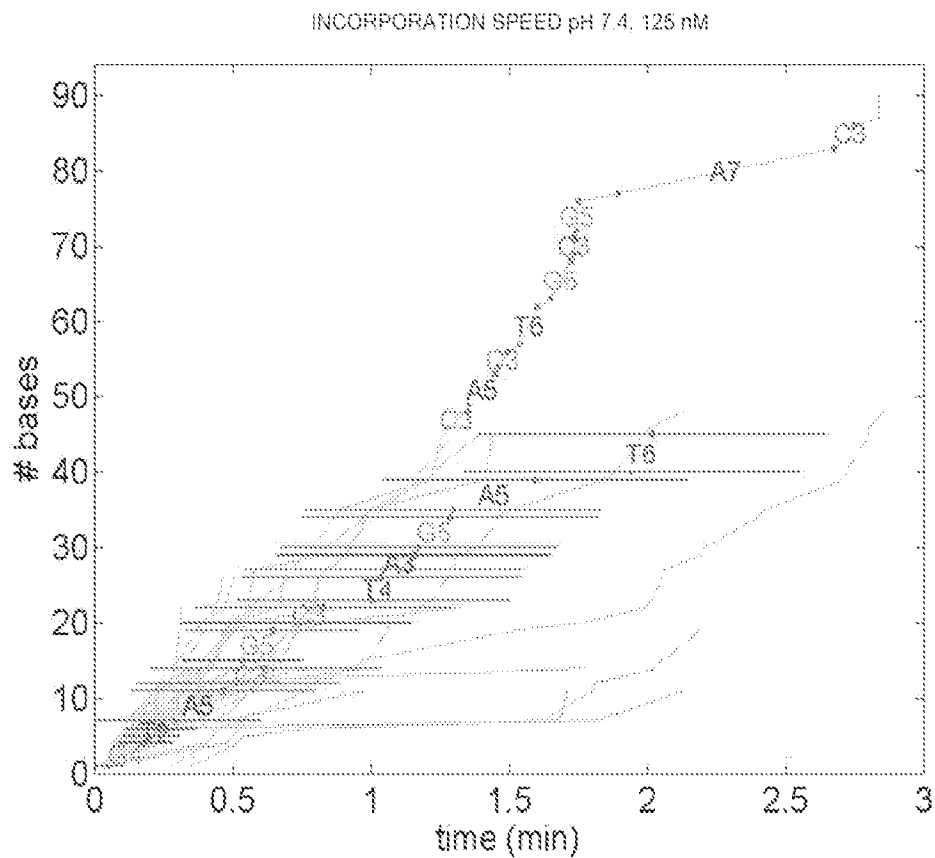
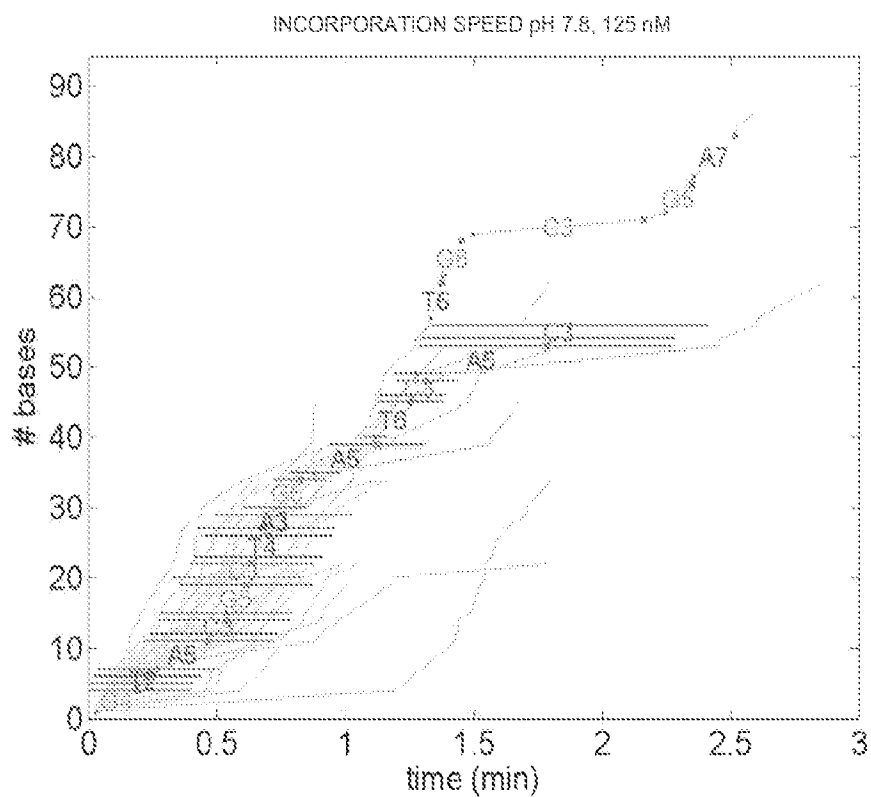


FIG. 12

**FIG. 13****FIG. 14**

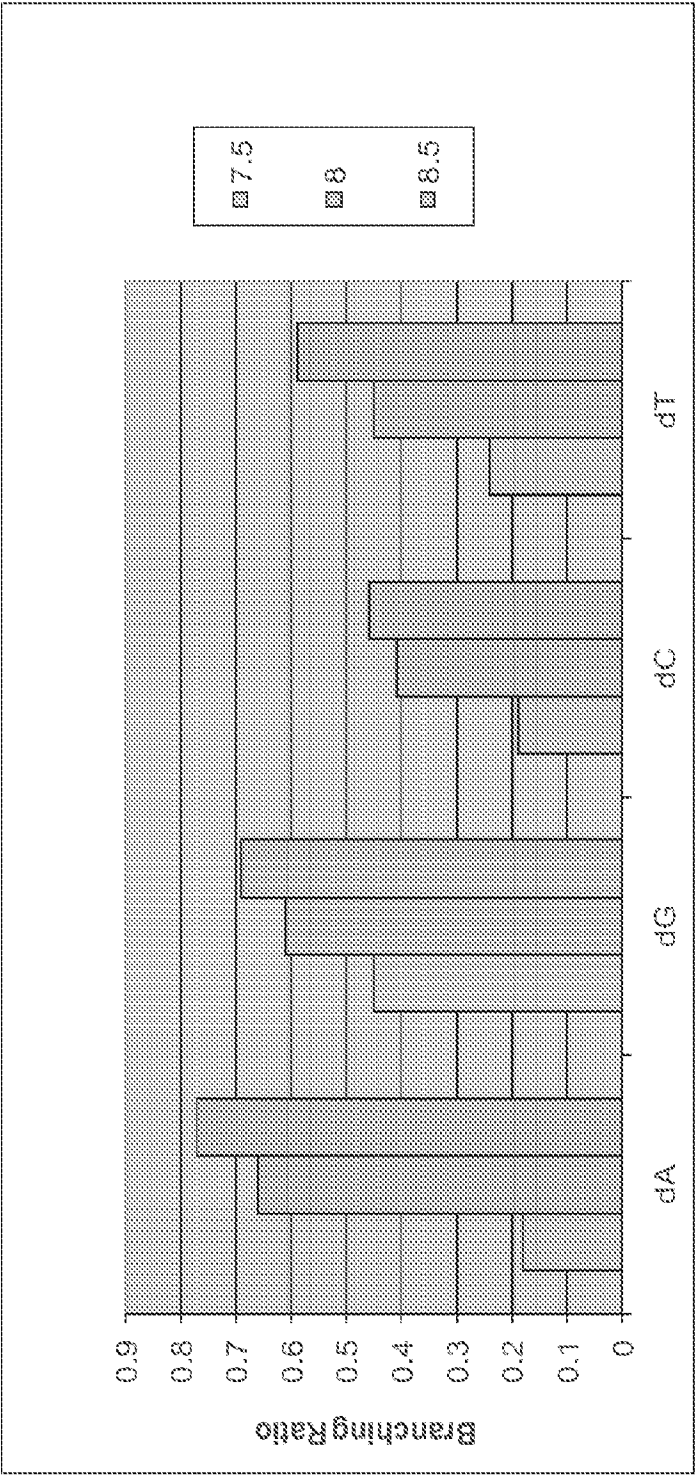


FIG. 15