

PATENT APPLICATION

NUCLEIC ACID SEQUENCING USING AFFINITY REAGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/442,263, filed January 4, 2017 and U.S. Provisional Application No. 62/490,511, filed April 26, 2017. The entire content of these applications is incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

[0002] The need for low cost, high-throughput, methods for nucleic acid sequencing and re-sequencing has led to the development “massively parallel sequencing” (MPS) technologies. One commonly used method for sequencing DNA is referred to as “sequencing-by-synthesis” (SBS), such as disclosed in Ronaghi et al., *Science*, 281:363-365, 1998; Li et al., *Proc. Natl. Acad. Sci. USA*, 100:414-419, 2003; Metzker, *Nat Rev Genet.* 11:31-46, 2010; Ju et al., *Proc. Natl. Acad. Sci. USA* 103:19635–19640, 2006; Bentley et al., *Nature* 456:53–59, 2008; and in U.S. Pat. Nos. 6,210,891, 6,828,100, 6,833,246, and 6,911,345, and U.S. Pat. Pub. N2016/0130647.

[0003] SBS requires the controlled (*i.e.*, one at a time) incorporation of the correct complementary nucleotide opposite the oligonucleotide being sequenced. This allows for accurate sequencing by adding nucleotides in multiple cycles as each nucleotide residue is sequenced one at a time, thus preventing an uncontrolled series of incorporations occurring. In one approach reversible terminator nucleotides (RTs) are used to determine the sequence of the DNA template. In the most commonly used SBS approach, each RT comprises a modified

Claims

1. A method for detecting of a 3'-O-reversible terminator deoxyribonucleotide incorporated at the 3' end of a primer extension product, wherein the reversible terminator deoxyribonucleotide comprises a nucleobase, a sugar moiety, and a cleavable blocking group, said method comprising:
 - (a) providing the primer extension product comprising the incorporated reversible terminator deoxyribonucleotide;
 - (b) combining the primer extension product from (a) with a first affinity reagent that binds to the incorporated reversible terminator deoxyribonucleotide, wherein the first affinity reagent binds to the nucleobase, the cleavable blocking group, or both, or to the nucleobase and 3'-OH moiety of the 3'-OH deoxyribonucleotide extension product, and
 - (c) detecting binding of the first affinity reagent to the incorporated reversible terminator deoxyribonucleotide.
2. The method of claim 1 where the first affinity reagent binds to an epitope comprising the sugar moiety.
3. The method of claim 1 wherein the nucleobase is a natural nucleobase selected from adenine (A), guanine (G), thymine (T), cytosine (C), and uracil (U).
4. The method of claim 3 wherein the first affinity reagent discriminates
 - (a) an incorporated reversible terminator comprising adenine (A) from an incorporated reversible terminator comprising deoxyribonucleotide guanine (G), thymine (T), or cytosine (C);
 - (b) an incorporated reversible terminator comprising G from an incorporated reversible terminator comprising T, C, or A;
 - (c) an incorporated reversible terminator comprising T from an incorporated reversible terminator comprising G, C, or A; or
 - (d) an incorporated reversible terminator comprising C from an incorporated reversible terminator comprising guanine G, T, or A.
5. The method of claim 1 wherein the nucleobase is an adenine analog (A'), a guanine analog (G'), a thymine analog (T'), or a cytosine analog (C'), and the first affinity reagent preferentially binds an incorporated nucleotide comprising the analog compared to binding an incorporated nucleotide comprising the corresponding natural nucleobase.
6. The method of claim 5 wherein the first affinity reagent binds to the cleavable blocking group.
7. The method of claim 1 wherein in step (c) the primer extension product is combined with 2, 3 or 4 affinity reagents with different binding specificities and one affinity reagent binds to the incorporated reversible terminator deoxyribonucleotide.
8. The method of claim 7 wherein the binding by said one affinity reagent identifies the nucleobase.
9. The method of any of claims 1 to 8, further comprising (d) removing the cleavable blocking group after step (c) to produce a 3'-OH deoxyribonucleotide.

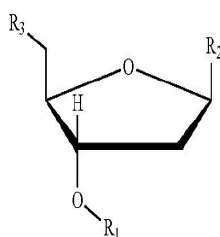
10. The method of any of claims 1 to 8, wherein the first affinity reagent is an antibody or an aptamer.
11. The method of any of claims 1 to 8, wherein the first affinity reagent is conjugated to or bound to a detectable label, and detecting binding of the affinity reagent to the incorporated reversible terminator deoxyribonucleotide in step (c) comprises detecting a signal from the detectable label.
12. The method of any of claims 1 to 8, wherein the first affinity reagent is not labeled and the step of detecting binding of the first affinity reagent to the incorporated reversible terminator deoxyribonucleotide comprises:
 - (i) binding one or more secondary affinity reagents to the first affinity reagent, wherein the secondary affinity reagents comprise a detectable label, and
 - (ii) detecting binding of the first affinity reagent to the incorporated reversible terminator deoxyribonucleotide comprises detecting a signal from the detectable label.
13. The method of claim 12 where the first affinity reagent and the secondary affinity reagent are combined prior to step (b).
14. The method of any of claims 1 to 8, wherein binding is detected in step (d) by detecting a fluorescence or chemiluminescence signal.
15. A method for performing a sequencing-by-synthesis reaction, said method comprising the steps of:
 - (a) providing a DNA array comprising a plurality of immobilized template nucleic acids comprising a plurality of different template sequences wherein the different template sequences of the are immobilized at different positions on the array;
 - (b) annealing oligonucleotide primers to the template nucleic acids wherein the oligonucleotide primers hybridize to predetermined positions on the template nucleic acids;
 - (c) combining the template nucleic acids and primers annealed thereto with a polymerase and four different reversible terminator deoxyribonucleotides each reversible terminator deoxyribonucleotide comprising a nucleobase (N), a sugar moiety, and a cleavable blocking group, wherein N is adenine (A) or an analog thereof (A'), guanine (G) or an analog thereof (G'), thymine (T) or an analog thereof (T'), and cytosine (C) or an analog thereof (C'), wherein at least one of said four different reversible terminator deoxyribonucleotides is unlabeled, under conditions in which a plurality of the oligonucleotide primers are extended by incorporation of a single reversible terminator deoxyribonucleotide each to produce a plurality of primer extension products each comprising a 3' terminus, some of which comprise A or A' incorporated at the 3' terminus, some of which comprise T or T' incorporated at the 3' terminus, some of which comprise G or G' incorporated at the 3' terminus, and some of which comprise C or C' incorporated at the 3' terminus;
 - (d) contacting the plurality of primer extension products with one or more first affinity reagents under conditions wherein each of said one or more first affinity reagents binds to only one of the four different incorporated reversible terminator deoxyribonucleotides, wherein the first affinity reagents bind to the nucleobase, the sugar moiety, the cleavable blocking group or a combination thereof, of said one of four incorporated reversible terminator deoxyribonucleotides;

(e) detecting the binding of the one or more first affinity reagents, wherein the binding of a first affinity reagent to a primer extension product comprising an incorporated reversible terminator deoxyribonucleotide identifies the nucleobase of the incorporated reversible terminator deoxyribonucleotide and the nucleobase of the template nucleotide complementary to the nucleobase of the incorporated reversible terminator deoxyribonucleotide.

16. The method of claim 15 wherein all of the four different reversible terminator deoxyribonucleotides are unlabeled.

17. A method for sequencing a nucleic acid, comprising:

(a) contacting a nucleic acid template comprising the nucleic acid, a nucleic acid primer complementary to a portion of said template, a polymerase, and an unlabeled RT of Formula I:



wherein R_1 is a 3'-O reversible cleavable blocking group;

R_2 is a nucleobase selected from adenine (A), cytosine (C), guanine (G), thymine (T), and analogues thereof; and

R_3 consists of one or more phosphates;

under conditions suitable for extending the primer by incorporating the unlabeled RT into a sequence complementary to the nucleic acid template, thereby producing an unlabeled extension product comprising the unlabeled RT;

(b) contacting the unlabeled extension product with an affinity reagent comprising a detectable label under conditions wherein the affinity reagent binds specifically to the unlabeled RT to produce a labeled extension product comprising the RT; and

(c) identifying the unlabeled RT in the labeled extension product to identify at least a portion of the sequence of said nucleic acid.

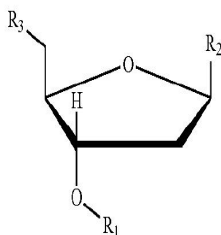
18. A DNA array comprising:

a plurality of different template DNA molecules immobilized at different locations on the array, wherein a plurality of the template DNA molecules comprise primer extension products annealed thereto wherein the primer extension products comprise 3' reversible terminator deoxyribonucleotides comprising A, T, G or C nucleobases or analogs thereof; and

affinity reagents specifically bound to at least some of the first reversible terminator deoxyribonucleotides.

19. A kit comprising:

(a) four types of unlabeled reversible terminator deoxyribonucleotides of Formula I:



wherein R_1 is a 3'-O cleavable blocking group;

R_2 is a nucleobase selected from adenine (A), cytosine (C), guanine (G), thymine (T), and analogues thereof and each type of reversible terminator deoxyribonucleotide comprises a different nucleobase; and

R_3 comprises of one or more phosphates;

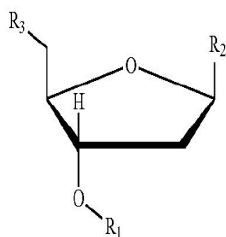
wherein R_1 is independently selected for each type of reversible terminator deoxyribonucleotide;

(b) a plurality of first affinity reagents wherein each first affinity reagent binds a different one of the reversible terminator deoxyribonucleotides when incorporated at the 3' terminus of a primer extension product; and

(c) packaging for (a) and (b).

20. The method of any of claims 1 to 8, wherein the primer extension product is on a DNA array comprising a plurality of primer extension products, wherein each primer extension product is hybridized to a template DNA, the method comprising:

(a) contacting the DNA array with a nucleic acid primer complementary to a portion of each of said template DNA molecules, a polymerase, and an unlabeled reversible terminator deoxyribonucleotide of Formula I:



wherein R_1 is a 3'-O cleavable blocking group;

R_2 is a nucleobase selected from adenine (A), cytosine (C), guanine (G), thymine (T), and analogues thereof; and

R_3 consists of one or more phosphates;

under conditions wherein the primer is extended to incorporate the unlabeled reversible terminator deoxyribonucleotide into a sequence complementary to at least some of said plurality of said template DNA molecules, thereby producing unlabeled extension products comprising the reversible terminator deoxyribonucleotide;

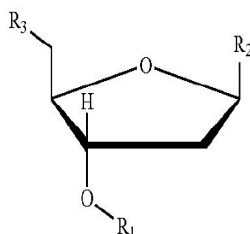
- (b) contacting the unlabeled extension products with an affinity reagent comprising a detectable label under conditions wherein the affinity reagent binds specifically to the reversible terminator nucleotide to produce labeled extension products comprising the reversible terminator deoxyribonucleotide; and
 - (c) identifying the reversible terminator deoxyribonucleotide in the labeled extension products thereby to identify at least a portion of the sequence of said nucleic acid.
- 21. The method of claim 15 wherein four reversible terminator deoxyribonucleotides are unlabeled and in step (d) four first affinity reagents are used, each specifically binding to one of the four incorporated reversible terminator deoxyribonucleotides.
- 22. The method of claim 15 wherein at least one of the four different reversible terminator deoxyribonucleotides comprises a naturally occurring nucleobase.
- 23. The method of claim 15, further comprising:
 - (f) removing the first affinity reagent(s).
- 24. The method of claim 23 further comprising:
 - (g) removing the cleavable blocking group to produce a 3'-OH deoxyribonucleotide, and repeating steps (c) to (g) for 25 or more cycles to identify additional nucleobases in the template,
 - wherein the primers annealed to the template nucleic acids in step (c) are primer extension products of a previous cycle.
- 25. The method of claim 24, wherein the first affinity reagents are antibodies.
- 26. The method of claim 24, wherein the nucleobase is a natural nucleobase selected from adenine (A), guanine (G), thymine (T) cytosine (C), and uracil (U).
- 27. The method of claim 15, wherein binding is detected in step (e) by detecting a fluorescence or chemiluminescence signal.
- 28. The method of claim 15, wherein the first affinity reagents are aptamers.
- 29. The method of claim 15, wherein the cleavable blocking group is selected from the group consisting of allyl, azidomethyl, aminoalkoxyl, 2-cyanoethyl, substituted alkyl, unsubstituted alkyl, substituted alkenyl, unsubstituted alkenyl, substituted alkynyl, unsubstituted alkynyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted heteroalkenyl, unsubstituted heteroalkenyl, substituted heteroalkynyl, unsubstituted heteroalkynyl, allenyl, cis-cyanoethenyl, trans-cyanoethenyl, cis-cyanofluoroethenyl, trans-cyanofluoroethenyl, cis-trifluoromethylethenyl, trans-trifluoromethylethenyl, biscyanoethenyl, bisfluoroethenyl, cis-propenyl, trans-propenyl, nitroethenyl, acetoethenyl, methylcarboethenyl, amidoethenyl, methylsulfoethenyl, methylsulfoethyl, formimidate, formhydroxymate, vinyloethenyl, ethylenoethenyl, cyanoethylenyl, nitroethylenyl, amidoethylenyl, amino, cyanoethenyl, cyanoethyl, alkoxy, acyl, methoxymethyl, aminoxyl, carbonyl, nitrobenzyl, coumarinyl, and nitronaphthalenyl.
- 30. The method of claim 29 wherein the cleavable blocking group is azidomethyl.

31. The method of claim 16 wherein in steps (d) and (e) two, three, or four first affinity reagents are used.
32. The method of claim 24, comprising removing the cleavable blocking group(s) and the affinity reagent(s) in the same reaction.
33. The method of claim 24, in which the first affinity reagent is removed without removing the cleavable blocking group.
34. The method of claim 33, comprising:
 - (i) detecting the binding of an affinity reagent to an incorporated reversible terminator deoxyribonucleotide;
 - (ii) removing the affinity reagent to without removing the cleavable blocking group of the incorporated reversible terminator deoxyribonucleotide;
 - (iii) reprobing the same incorporated reversible terminator deoxyribonucleotide by detecting the binding of an affinity reagent.
35. The method of claim 34 wherein one affinity reagent of the affinity reagent in (i) and the affinity reagent in (ii) is an antibody and one affinity reagent of the affinity reagent in (i) and the affinity reagent in (ii) is an aptamer.
36. The method of claim 17 wherein multiple cycles of sequencing-by-synthesis are carried out, wherein each cycle comprises contacting the nucleic acid template with four different reversible terminator nucleotides of Formula I, each containing a different nucleobase selected from adenine (A), cytosine (C), guanine (G), thymine (T), and analogues thereof, wherein at least one of the four different reversible terminator nucleotides is an unlabeled RT, and wherein the conditions in step (a) are suitable for incorporation of labeled and unlabeled reversible terminator nucleotides.
37. The method of claim 17 wherein at least 25 cycles of sequencing-by-synthesis are carried out.
38. The method of claim 17 wherein all four of the four different reversible terminator nucleotides are unlabeled.
39. The method of claim 17 wherein at least one of the reversible terminator nucleotides is fluorescently labeled.

40. The method of claim 17 wherein the cleavable blocking group is selected from the group consisting of allyl, azidomethyl, aminoalkoxyl, 2-cyanoethyl, substituted alkyl, unsubstituted alkyl, substituted alkenyl, unsubstituted alkenyl, substituted alkynyl, unsubstituted alkynyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted heteroalkenyl, unsubstituted heteroalkenyl, substituted heteroalkynyl, unsubstituted heteroalkynyl, allenyl, cis-cyanoethenyl, trans-cyanoethenyl, cis-cyanofluoroethenyl, trans-cyanofluoroethenyl, cis-trifluoromethylethenyl, trans-trifluoromethylethenyl, biscyanoethenyl, bisfluoroethenyl, cis-propenyl, trans-propenyl, nitroethenyl, acetoethenyl, methylcarboethenyl, amidoethenyl, methylsulfoethenyl, methylsulfoethyl, formimidate, formhydroxymate, vinyloethenyl, ethylenoethenyl, cyanoethylenyl, nitroethylenyl, amidoethylenyl, amino, cyanoethenyl, cyanoethyl, alkoxy, acyl, methoxymethyl, aminoxyl, carbonyl, nitrobenzyl, coumarinyl, and nitronaphthalenyl.
41. The method of claim 40 wherein the cleavable blocking group is azidomethyl.
42. The DNA array of claim 18 wherein the template DNA molecules immobilized at different locations on the array are DNA nanoballs or are clonal populations of amplicons produced using bridge amplification.
43. The kit of claim 19 wherein the cleavable blocking group of at least one of the types of unlabeled reversible terminator nucleotides is azidomethyl.
44. A method for detecting a 3'-O-reversible terminator deoxyribonucleotide incorporated at the 3' end of a primer extension product, wherein the reversible terminator deoxyribonucleotide comprises a nucleobase, a sugar moiety, and a cleavable blocking group, said method comprising:
 - (a) providing the primer extension product comprising the incorporated reversible terminator deoxyribonucleotide;
 - (b) removing the cleavable blocking group of the incorporated reversible terminator deoxyribonucleotide to produce a 3'-OH deoxyribonucleotide moiety of the primer extension product; and then
 - (c) combining the primer extension product from (b) with a first affinity reagent that binds to the incorporated reversible terminator deoxyribonucleotide, wherein the first affinity reagent binds to the nucleobase and 3'-OH moiety of the 3'-OH primer extension product, and
 - (d) detecting binding of the first affinity reagent to the incorporated reversible terminator deoxyribonucleotide.
45. The method of claim 44 wherein the first affinity reagent is an antibody or an aptamer.
46. The method of claim 44 wherein the nucleobase is a natural nucleobase selected from adenine (A), guanine (G), thymine (T), cytosine (C), and uracil (U).

47. A method for sequencing a nucleic acid, comprising:

- (a) providing a DNA array comprising (i) a plurality of template DNA molecules, each template DNA molecule comprising a primer binding site and a fragment of the nucleic acid, wherein each of said plurality of template DNA molecules is attached at a position of the array,
- (b) contacting the DNA array with a nucleic acid primer complementary to the primer binding site, a polymerase, and an unlabeled reversible terminator nucleotide (RT) of Formula I:



wherein R₁ is a 3'-O cleavable blocking group;

R₂ is a nucleobase selected from adenine (A), cytosine (C), guanine (G), thymine (T), and analogues thereof; and

R₃ consists of one or more phosphates;

under conditions wherein the primer hybridizes to primer binding sites of the template DNA molecules and wherein at least some hybridized primers are extended to incorporate the unlabeled RT into a sequence complementary to the template DNA molecule, thereby producing unlabeled extension products comprising the unlabeled RT;

(c) contacting the unlabeled extension products with an affinity reagent under conditions wherein the affinity reagent binds specifically to the incorporated RT, wherein the affinity reagent is directly or indirectly associated with a detectable label to produce labeled extension products comprising the RT; and

(d) identifying the RT in the labeled extension products thereby to identify at least a portion of the sequence of said nucleic acid.

48. The method of claim 47 comprising:

(a) contacting the DNA array with a nucleic acid primer complementary to a portion of each of said template DNA molecules, a polymerase, and a set of unlabeled RTs of Formula I that comprises a first RT wherein R₂ is adenine (A), a second RT wherein R₂ is thymine (T), a third RT wherein R₂ is cytosine (C), and a fourth RT wherein R₂ is guanine (G), under conditions wherein the primer is extended to incorporate the unlabeled RTs into a sequence complementary to at least some of said plurality of said template DNA molecules, thereby producing unlabeled extension products comprising the RTs;

(b) contacting the unlabeled extension products with a set of affinity reagents under conditions wherein the set of affinity reagents binds specifically to the RTs to produce labeled extension products comprising the RTs, wherein:

(i) the set of affinity reagents comprises a first affinity reagent that binds specifically to the first RT, a second affinity reagent that binds specifically to the second RT, a third affinity reagent that binds specifically to the third RT, and, optionally, a fourth affinity reagent that binds specifically to the fourth RT; and

(ii) at least the first, second, and third affinity reagents comprise detectable labels;

and

(c) identifying the RTs in the labeled extension products by identifying the label of the labeled affinity reagents bound to the RTs at their respective positions on the array to identify at least a portion of the sequence of said nucleic acid.

49. The method of claim 48 further comprising:

- (d) removing the blocking group from the RT to produce a 3'-OH; and
- (e) removing the affinity reagent from the RT.

50. The method of any of claims 47 to 49, wherein the template DNA molecules immobilized at different locations on the array are DNA nanoballs or are clonal populations of amplicons produced using bridge amplification.