

WHAT IS CLAIMED IS:

1. A method for determining the sequence of a target polynucleotide, comprising:
 - (a) contacting a primer polynucleotide with a mixture comprising one or more of a first type of nucleotide, a second type of nucleotide, a third type of nucleotide, and a fourth type of nucleotide, wherein the primer polynucleotide is complementary to at least a portion of the target polynucleotide;
 - (b) incorporating one type of nucleotide from the mixture to the primer polynucleotide to produce an extended primer polynucleotide;
 - (c) performing a first imaging event using a first excitation light source and collecting a first emission signal from the extended primer polynucleotide with a first emission filter; and
 - (d) performing a second imaging event using a second excitation light source and collecting a second emission signal from the extended primer polynucleotide with a second emission filter;wherein one of the first excitation light source and the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the other one of the first excitation light source and the second excitation light source has a wavelength of about 450 nm to about 460 nm; and
wherein one of the first emission filter and the second emission filter has a detection wavelength of about 415 nm to about 450 nm, and the other one of the first emission filter and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.
2. The method of claim 1, wherein the first type of nucleotide is labeled with a first detectable label that is excitable by the first excitation light source and detectable by the first emission filter.
3. The method of claim 1 or 2, wherein the second type of nucleotide is labeled with a second detectable label that is excitable by the second excitation light source and detectable by the second emission filter, and wherein the second type of detectable label is spectrally distinguishable from the first type of detectable label.
4. The method of any one of claims 1 to 3, wherein the third type of nucleotide is labeled both with a first detectable label and a second detectable label, and the third type of nucleotide is excitable by both the first excitation light source and the second excitation light source.

5. The method of any one of claims 1 to 3, wherein the third type of nucleotide comprises a mixture of a third type of nucleotide labeled with a third label and a third type of nucleotide labeled with a fourth label, wherein the third label is excitable by the first excitation light source and detectable by the first emission filter, and wherein the fourth label is excitable by the second excitation light source and detectable by the second emission filter.

6. The method of claim 1, wherein each of the first type, the second type and the third type of nucleotide is unlabeled, and the method further comprising: contacting the extended primer polynucleotide with a set of affinity reagents prior to the first imaging event, wherein at least one affinity reagent in the set binds specifically to the incorporated first type, second type, or third type of nucleotide.

7. The method of claim 6, wherein the set of affinity reagents comprises: a first affinity reagent that binds specifically to the first type of nucleotide, a second affinity reagent that binds specifically to the second type of nucleotide.

8. The method of claim 7, wherein the first affinity reagent comprises one or more first detectable labels that are excitable by the first excitation light source and detectable by the first emission filter, the second affinity reagent comprises one or more second detectable labels that are excitable by the second excitation light source and detectable by the second emission filter, and wherein the first detectable label is spectrally distinguishable from the second detectable label.

9. The method of claim 7 or 8, wherein both the first affinity reagent and the second affinity reagent bind specifically to the third type of nucleotide.

10. The method of claim 7 or 8, wherein the set of affinity reagents further comprises a third affinity reagent that binds specifically to the third type of nucleotide, and wherein the third affinity reagent comprises one or more third detectable labels that are excitable by the first excitation light source and detectable by the first emission filter, and one or more fourth detectable labels that are excitable by the second excitation light source and detectable by the second emission filter.

11. The method of any one of claims 7 to 10, wherein the first type of nucleotide comprises a first hapten, and the first affinity reagent comprises a first hapten-binding partner that specifically binds to the first hapten.

12. The method of claim 11, wherein the first hapten comprises a biotin moiety and the first hapten-binding partner comprises streptavidin.

13. The method of any one of claims 7 to 12, wherein the second type of nucleotide comprises a second hapten, and the second affinity reagent comprises a second hapten-binding partner that specifically binds to the second hapten.

14. The method of claim 13, wherein the second hapten comprises a chloroalkyl group and the second hapten-binding partner comprises HaloTag®.

15. The method of claim 1, wherein the first type of nucleotide is labeled with a first detectable label, the second type of nucleotide is unlabeled, the third type of nucleotide is both unlabeled and labeled with the first detectable label, and the first detectable label is excitable by the first excitation light source and detectable by the first emission filter, and the method further comprising: contacting the extended primer polynucleotide with an affinity reagent prior to the first imaging event, wherein the affinity reagent binds specifically to the second type of unlabeled nucleotide or the third type of unlabeled nucleotide.

16. The method of claim 15, wherein the affinity reagent comprises one or more second detectable labels that are excitable by the second excitation light source and detectable by the second emission filter.

17. The method of claim 16, wherein the affinity reagent comprises streptavidin, and both the second type of nucleotide and the third type unlabeled nucleotide comprise a biotin moiety.

18. The method of claim 1, wherein the first type of nucleotide is unlabeled, the second type of nucleotide is labeled with a second detectable label, the third type of nucleotide is both unlabeled and labeled with the second detectable label, and the second detectable label is excitable by the second excitation light source and detectable by the second emission filter, and the method further comprising: contacting the extended primer polynucleotide with an affinity reagent prior to the first imaging event, wherein the affinity reagent binds specifically to the first type of unlabeled nucleotide or the third type of unlabeled nucleotide.

19. The method of claim 18, wherein the affinity reagent comprises one or more first detectable labels that are excitable by the first excitation light source and detectable by the first emission filter.

20. The method of claim 19, wherein the affinity reagent comprises streptavidin, and both the first type of nucleotide and the third type unlabeled nucleotide comprise a biotin moiety.

21. The method of any one of claims 1 to 20, wherein the fourth type of nucleotide is unlabeled (dark), or is labeled with a fluorescent moiety that has no emission from either the first imaging event or the second imaging event.

22. The method of any one of claims 1 to 21, wherein the four types of nucleotide comprise dATP, dCTP, dGTP and dTTP or dUTP, or non-natural nucleotide analogs thereof.

23. The method of claim 22, wherein each of the four types of nucleotide in the mixture has a 3' hydroxyl blocking group.

24. The method of any one of claims 1 to 23, further comprising: (e) removing the 3' hydroxyl blocking group from the incorporated nucleotide after the second imaging event, and prior to the next sequencing cycle.

25. The method of claim 24, further comprising:
repeating steps (a)-(e) for multiple cycles; and
determining the sequence of the target polynucleotide based on the sequentially incorporated nucleotides.

26. The method of claim 25, wherein steps (a)-(e) are repeated for at least 50 cycles.

27. The method of any one of claims 1 to 26, wherein each of the first excitation light source and the second excitation light source comprises a laser, a light-emitting diode (LED), or a combination thereof.

28. The method of any one of claims 1 to 27, wherein the first excitation light source has a wavelength of about 350 nm to about 410 nm, and the first emission filter has a detection wavelength of about 415 nm to about 450 nm.

29. The method of claim 28, wherein the second excitation light source has a wavelength of about 450 nm to about 460 nm, and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

30. The method of any one of claims 1 to 27, wherein the first excitation light source has a wavelength of about 450 nm to about 460 nm, and the first emission filter has a detection wavelength of about 480 nm to about 525 nm.

31. The method of claim 30, wherein the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the second emission filter has a detection wavelength of about 415 nm to about 450 nm.

32. The method of any one of claims 1 to 31, wherein the extended primer polynucleotide is in a buffer solution comprising one or more antioxidants during the first imaging event and the second imaging event.

33. The method of any one of claims 1 to 32, wherein the target polynucleotide is immobilized to a solid support.

34. The method of claim 33, wherein the solid support comprises a plurality of immobilized target polynucleotides.

35. The method of claim 33 or 34, wherein the solid support comprises a flow cell.

36. The method of claim 35, wherein the flow cell is a patterned flow cell comprises a plurality of nanowells and each nanowell comprises one immobilized target polynucleotide.

37. The method of any one of claims 33 to 36, wherein the density of the immobilized target polynucleotides on the solid support is from about 100k/mm² to about 300k/mm².

38. A kit for sequencing application, comprising:

- a first type of nucleotide labeled with a first detectable label;
- a second type of nucleotide labeled with a second detectable label;
- a third type of nucleotide labeled with the first detectable label; and
- a third type of nucleotide labeled with the second detectable label;

wherein the first detectable label and the second detectable label are spectrally distinguishable from one another, the first detectable label is excitable by a first light source and detectable by a first emission filter, and the second detectable label is excitable by a second light source and detectable by a second emission filter;

wherein one of the first excitation light source and the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the other one of the first

excitation light source and the second excitation light source has a wavelength of about 450 nm to about 460 nm; and

wherein one of the first emission filter and the second emission filter has a detection wavelength of about 415 nm to about 450 nm, and the other one of the first emission filter and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

39. A kit for sequencing application, comprising:

- a first type of nucleotide labeled with a first detectable label;
- a second type of nucleotide labeled with a second detectable label;
- a third type of nucleotide labeled with a third detectable label; and
- a fourth type of nucleotide labeled with a fourth detectable label;

wherein the first detectable label and the second detectable label are spectrally distinguishable from one another, the first detectable label is excitable by a first light source and detectable by a first emission filter, and the second detectable label is excitable by a second light source and detectable by a second emission filter;

wherein the third detectable label and the fourth detectable label are spectrally distinguishable from one another, the third detectable label is excitable by the first light source and detectable by the first emission filter, and the fourth detectable label is excitable by the second light source and detectable by the second emission filter;

wherein one of the first excitation light source and the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the other one of the first excitation light source and the second excitation light source has a wavelength of about 450 nm to about 460 nm; and

wherein one of the first emission filter and the second emission filter has a detection wavelength of about 415 nm to about 450 nm, and the other one of the first emission filter and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

40. A kit for sequencing application, comprising:

- a first type of unlabeled nucleotide;
- a second type of unlabeled nucleotide;
- a third type of unlabeled nucleotide; and
- a set of affinity reagents comprising:

a first affinity reagent that binds specifically to the first type of unlabeled nucleotide; and

a second affinity reagent that binds specifically to the second type of unlabeled nucleotide;

wherein the first affinity reagent comprises one or more first detectable labels that are excitable by a first excitation light source and detectable by a first emission filter, the second affinity reagent comprises one or more second detectable labels that are excitable by a second excitation light source and detectable by a second emission filter, and wherein the first detectable label is spectrally distinguishable from the second detectable label;

wherein one of the first excitation light source and the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the other one of the first excitation light source and the second excitation light source has a wavelength of about 450 nm to about 460 nm; and

wherein one of the first emission filter and the second emission filter has a detection wavelength of about 415 nm to about 450 nm, and the other one of the first emission filter and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

41. The kit of claim 40, wherein the first type of nucleotide comprises a first hapten, and the first affinity reagent comprises a first hapten-binding partner that specifically binds to the first hapten.

42. The kit of claim 41, wherein the first hapten comprises a biotin moiety and the first hapten-binding partner comprises streptavidin.

43. The kit of any one of claims 40 to 42, wherein the second type of nucleotide comprises a second hapten, and the second affinity reagent comprises a second hapten-binding partner that specifically binds to the second hapten.

44. The kit of claim 43, wherein the second hapten comprises a chloroalkyl group and the second hapten-binding partner comprises HaloTag®.

45. The kit of any one of claims 39 to 44, wherein the third type of nucleotide comprises both a first hapten and a second hapten, and wherein both the first affinity reagent and the second affinity reagent bind specifically to the third type of unlabeled nucleotide.

46. The kit of any one of claims 40 to 44, wherein the set of affinity reagents further comprises a third affinity reagent that binds specifically to the third type of nucleotide, and wherein the third affinity reagent comprises one or more third detectable labels that are excitable by the first excitation light source and detectable by the first emission filter, and one or more fourth detectable labels that are excitable by the second excitation light source and detectable by the second emission filter.

47. A kit for sequencing application, comprising:

a first type of nucleotide either unlabeled or labeled with a first detectable label;

a second type of nucleotide either unlabeled or labeled with a second detectable label, wherein one of the first type of nucleotide and the second type of nucleotide is unlabeled;

a third type of unlabeled nucleotide, and a third type of nucleotide labeled with the same detectable label as either the first or the second type of nucleotide, wherein the first detectable label and the second detectable label are spectrally distinguishable from one another, the first detectable label is excitable by a first light source and detectable by a first emission filter, and the second detectable label is excitable by a second light source and detectable by a second emission filter; and

an affinity reagent comprising either a first affinity reagent that binds specifically to the third type of unlabeled nucleotide and the first type of nucleotide if the first type of nucleotide is unlabeled, or a second affinity reagent that binds specifically to the third type of unlabeled nucleotide and the second type of nucleotide if the second type of nucleotide is unlabeled, wherein the first affinity reagent comprises one or more first detectable labels and the second affinity reagent comprises one or more second detectable labels;

wherein one of the first excitation light source and the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the other one of the first excitation light source and the second excitation light source has a wavelength of about 450 nm to about 460 nm; and

wherein one of the first emission filter and the second emission filter has a detection wavelength of about 415 nm to about 450 nm, and the other one of the first emission filter and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

48. The kit of claim 47, wherein the first type of nucleotide is unlabeled, the second type of nucleotide is labeled with a second detectable label, the third type of nucleotide is both

unlabeled and labeled with a second detectable label, and the affinity reagent is the first affinity reagent that binds specifically to the first type of nucleotide and the third type of unlabeled nucleotide, and wherein the first affinity reagent comprises one or more first detectable labels.

49. The kits of claim 47, wherein the first type of nucleotide is labeled with a first detectable label, the second type of nucleotide is unlabeled, the third type of nucleotide is both unlabeled and labeled with a second detectable label, and the affinity reagent is the second affinity reagent that binds specifically to the second type of nucleotide and the third type of unlabeled nucleotide, and wherein the second affinity reagent comprises one or more second detectable labels.

50. The kits of any one of claims 47 to 49, wherein when either the first type or the second type of nucleotide is unlabeled, such unlabeled nucleotide independently comprises a hapten.

51. The kit of claim 50, wherein the affinity reagent comprises a hapten-binding partner that specifically binds to the hapten in the unlabeled nucleotide.

52. The kit of claim 50 or 51, wherein the hapten comprises a biotin moiety or a chloroalkyl group.

53. The kit of claim 51 or 52, wherein the hapten-binding partner comprises streptavidin or HaloTag®.

54. The kit of any one of claims 38 to 53, wherein the first excitation light source has a wavelength of about 350 nm to about 410 nm, the first emission filter has a detection wavelength of about 415 nm to about 450 nm; the second excitation light source has a wavelength of about 450 nm to about 460 nm, and the second emission filter has a detection wavelength of about 480 nm to about 525 nm.

55. The kit of any one of claims 38 to 53, wherein the first excitation light source has a wavelength of about 450 nm to about 460 nm, the first emission filter has a detection wavelength of about 480 nm to about 525nm; the second excitation light source has a wavelength of about 350 nm to about 410 nm, and the second emission filter has a detection wavelength of about 415 nm to about 450 nm.

56. The kit of any one of claims 38 to 55, further comprising a fourth type of nucleotide, and wherein the fourth type of nucleotide is unlabeled (dark).

57. The kit of any one of claims 38 to 56, further comprising a DNA polymerase and one or more buffer compositions.

58. The kit of claim 57, wherein at least one buffer composition comprises one or more antioxidants for reducing DNA photo damage caused by the first excitation light source and/or the second excitation light source.

59. The kit of any one of claims 38 to 58, further comprising one or more solid support comprising a plurality of immobilized oligonucleotides, wherein the density of the immobilized oligonucleotides on the solid support is from about 100k/mm² to about 300k/mm².