

CLAIMS

1. A kit for sequencing application, comprising:
 - a first type of unlabeled nucleotide comprising a first hapten;
 - a second type of unlabeled nucleotide comprising a second hapten;
 - a third type of unlabeled nucleotide;
 - a set of affinity reagents comprising:
 - a first affinity reagent comprising a first hapten-binding partner that is capable of specific binding to the first type of unlabeled nucleotide; and
 - a second affinity reagent comprising a second hapten-binding partner that is capable of specific binding 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, the second affinity reagent comprises one or more second detectable labels that are excitable by a second excitation light source, and wherein the first detectable label is spectrally distinguishable from the second detectable label; and
 - a fourth type of unlabeled nucleotide, wherein the fourth type of unlabeled nucleotide is not capable of specific binding to any affinity reagents;
 - wherein 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 the other one of the first excitation light source and the second excitation light source has a wavelength of about 520 nm to about 540 nm.
2. The kit of claim 1, wherein the first hapten is covalently attached to the nucleobase of the first type of unlabeled nucleotide via a cleavable linker.
3. The kit of claim 1 or 2, wherein the second hapten is covalently attached to the nucleobase of the second type of unlabeled nucleotide via a cleavable linker.
4. The kit of any one of claims 1 to 3, wherein the third type of unlabeled nucleotide comprises a mixture of the third type of unlabeled nucleotide comprising the first hapten and the third type of unlabeled nucleotide comprising the second hapten, and wherein both the first affinity reagent and the second affinity reagent are capable of specific binding to the third type of

unlabeled nucleotide; optionally wherein each of the first hapten and the second hapten is covalently attached to the nucleobase of the third type of nucleotide via a cleavable linker.

5. The kit of claim 4, wherein the first hapten comprises a biotin moiety and the first hapten-binding partner comprises an avidin; optionally wherein:

- i) the avidin is streptavidin or neutravidin; and/or
- ii) one or more biotin binding sites of the avidin are blocked by a biotin moiety-containing molecule or an analog thereof; and/or

- iii) the second hapten comprises a dinitrophenyl (DNP) moiety and the second hapten-binding partner comprises an anti-DNP antibody, optionally wherein the third type of unlabeled nucleotide comprises a mixture of a third type of unlabeled nucleotide comprising a biotin moiety, and a third type of unlabeled nucleotide comprising a DNP moiety; and/or

- iv) the second hapten comprises a digoxigenin (DIG) moiety and the second hapten-binding partner comprises an anti-DIG antibody, optionally wherein the third type of unlabeled nucleotide comprises a mixture of a third type of unlabeled nucleotide comprising a biotin moiety, and a third type of unlabeled nucleotide comprising a DIG moiety.

6. The kit of any one of claims 1 to 3, wherein the third type of unlabeled nucleotide comprises a third hapten, and the set of affinity reagents further comprises a third affinity reagent comprising a third hapten-binding partner that is capable of specific binding to the third hapten.

7. The kit of claim 6, wherein:

- i) the third hapten is covalently attached to the nucleobase of the third type of unlabeled nucleotide via a cleavable linker; and/or

- ii) the first hapten comprises a biotin moiety and the first hapten-binding partner comprises an avidin, optionally wherein the avidin is streptavidin or neutravidin; and/or

- iii) one or more biotin binding sites of the avidin are blocked by a biotin moiety-containing molecule or an analog thereof; and/or

- iv) one of the second hapten and the third hapten comprises a DNP moiety, and the other one of the second hapten and the third hapten comprises a DIG moiety.

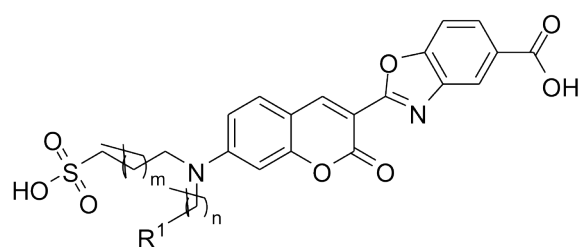
8. The kit of claim 6 or claim 7, wherein one of the first hapten and the second hapten comprises a DNP moiety, and the other one of the first hapten and the second hapten comprises a DIG moiety, optionally wherein the third hapten comprises a biotin moiety and the third

hapten-binding partner comprises an avidin, further optionally wherein the avidin is streptavidin or neutravidin and/or wherein one or more biotin binding sites of the avidin are blocked by a biotin moiety-containing molecule or an analog thereof.

9. The kit of any one of claims 6 to 8, wherein the third affinity reagent comprises one or more first detectable labels and one or more second detectable labels, optionally wherein the third affinity reagent comprises a multi-dye labeled protein assembly system.

10. The kit of any one of claims 1 to 9, wherein each of the first type, second type, third type and fourth type of unlabeled nucleotide comprises a 3' blocking group.

11. The kit of any one of claims 1 to 10, wherein the first detectable label is a dye having the structure of Formula (I):



R¹ is -C(O)OC₁-C₆ alkyl, -SO₃H, -OH, optionally substituted 5 to 10 membered heteroaryl, optionally substituted 3 to 10 membered heterocyclyl, optionally substituted phenyl, or -OR^x;

R^x is optionally substituted 5 to 10 membered heteroaryl, optionally substituted 3 to 10 membered heterocyclyl, or optionally substituted phenyl;

m is an integer of 1, 2 or 3; and

n is an integer of 1, 2, 3, 4 or 5.

12. The kit of any one of claims 1 to 11, further comprising a DNA polymerase and one or more buffer compositions.

13. A method of determining the sequences of a plurality of different target polynucleotides, comprising:

(a) contacting a solid support with a solution comprising sequencing primers under hybridization conditions, wherein the solid support comprises a plurality of

different target polynucleotides immobilized thereon; and the sequencing primers are complementary to at least a portion of the target polynucleotides;

(b) contacting the solid support with an aqueous solution comprising DNA polymerase and one or more of four different types of unlabeled nucleotides under conditions suitable for DNA polymerase-mediated primer extension, and incorporating one type of nucleotides into the sequencing primers to produce extended copy polynucleotides; wherein

each of the four types of nucleotides comprises a 3' blocking group;

a first type of unlabeled nucleotide comprises a first hapten;

a second type of unlabeled nucleotide comprises a second hapten;

a third type of nucleotide is unlabeled; and

a fourth type of nucleotide is unlabeled and the fourth type of unlabeled nucleotide is not capable of specific binding to any affinity reagents;

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

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

(e) removing the 3' blocking group of the incorporated nucleotides;

wherein the set of affinity reagents comprises:

a first affinity reagent comprising a first hapten-binding partner that is capable of specific binding to the first type of unlabeled nucleotides; and

a second affinity reagent comprising a second hapten-binding partner that is capable of specific binding to the second type of unlabeled nucleotides;

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

wherein 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 the other one of the first excitation light source and the second excitation light source has a wavelength of about 520 nm to about 540 nm.

14. The method of claim 13, wherein the first hapten is covalently attached to the nucleobase of the first type of unlabeled nucleotide via a cleavable linker.

15. The method of claim 13 or 14, wherein the second hapten is covalently attached to the nucleobase of the second type of unlabeled nucleotide via a cleavable linker.

16. The method of any one of claims 13 to 14, wherein the third type of unlabeled nucleotide comprises a mixture of the third type of unlabeled nucleotide comprising the first hapten and the third type of unlabeled nucleotide comprising the second hapten, and wherein both the first affinity reagent and the second affinity reagent are capable of specific binding to the third type of unlabeled nucleotide, optionally wherein each of the first hapten and the second hapten is covalently attached to the nucleobase of the third type of nucleotide via a cleavable linker.

17. The method of claim 16, wherein:

i) the first hapten comprises a biotin moiety and the first hapten-binding partner comprises avidin, optionally wherein the avidin is streptavidin or neutravidin; and/or

ii) one or more biotin binding sites of the avidin are blocked by a biotin moiety-containing molecule or an analog thereof; and/or

iii) the second hapten comprises a DNP moiety and the second hapten-binding partner comprises an anti-DNP antibody, optionally wherein the third type of unlabeled nucleotide comprises a mixture of a third type of unlabeled nucleotide comprising a biotin moiety, and a third type of unlabeled nucleotide comprising a DNP moiety; and/or

iv) the second hapten comprises a DIG moiety and the second hapten-binding partner comprises an anti-DIG antibody, optionally wherein the third type of unlabeled nucleotide comprises a mixture of a third type of unlabeled nucleotide comprising a biotin moiety, and a third type of unlabeled nucleotide comprising a DIG moiety.

18. The method of any one of claims 13 to 17, wherein:

i) the third type of unlabeled nucleotide comprises a third hapten, and the set of affinity reagents further comprises a third affinity reagent comprising a third hapten-binding partner that is capable of specific binding to the third hapten, optionally wherein the third hapten is covalently attached to the nucleobase of the third type of unlabeled nucleotide via a cleavable linker; and/or

ii) the first hapten comprises a biotin moiety and the first hapten-binding partner comprises an avidin, optionally wherein the avidin is streptavidin or neutravidin, further optionally wherein one of the second hapten and the third hapten comprises a DNP moiety, and the other one of the second hapten and the third hapten comprises a DIG moiety; and/or

iii) one of the first hapten and the second hapten comprises a DNP moiety, and the other one of the first hapten and the second hapten comprises a DIG moiety, optionally wherein the third hapten comprises a biotin moiety and the third hapten-binding partner comprises an avidin, optionally wherein the avidin is streptavidin or neutravidin, further optionally wherein one or more biotin binding sites of the avidin are blocked by a biotin moiety-containing molecule or an analog thereof; and/or

iv) wherein the third affinity reagent comprises one or more first detectable labels and one or more second detectable labels, optionally wherein the third affinity reagent comprises a multi-dye labeled protein assembly system.

19. The method of any one of claims 13 to 18, wherein step (e) also removes the detectable labels of the incorporated nucleotides, optionally wherein the detectable labels and the 3' blocking groups of the incorporated nucleotides are removed in a single chemical reaction.

20. The method of any one of claims 13 to 19, further comprising (f) washing the solid support with an aqueous wash solution, optionally wherein steps (b) to (f) are repeated at least 50, 100, 150, 200, 250, 300, 400 or 500 cycles to determine the target polynucleotide sequences.

21. The method of any one of claims 13 to 20, wherein:

i) the four types of nucleotides comprise dATP, dCTP, dGTP and dTTP or dUTP, or non-natural nucleotide analogs thereof; and/or

ii) the density of the immobilized target polynucleotides on the solid support is at least about 50,000/mm²; and/or

iii) the method reduces or eliminates sequence context effect.