

WHAT IS CLAIMED IS:

1. A method for identifying, in a sample, one or more nucleic acid molecules containing a target nucleotide sequence differing from nucleotide sequences in other nucleic acid molecules in the sample, or other samples, by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues, said method comprising:

providing a sample containing one or more nucleic acid molecules potentially containing the target nucleotide sequence differing from the nucleotide sequences in other nucleic acid molecules by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues;

providing one or more enzymes capable of digesting deoxyuracil (dU) containing nucleic acid molecules present in the sample;

providing one or more primary oligonucleotide primer sets, each primary oligonucleotide primer set comprising (a) a first primary oligonucleotide primer that comprises a nucleotide sequence that is complementary to a sequence adjacent to the target nucleotide sequence and (b) a second primary oligonucleotide primer that comprises a nucleotide sequence that is complementary to a portion of an extension product formed from the first primary oligonucleotide primer;

blending the sample, the one or more primary oligonucleotide primer sets, the one or more enzymes capable of digesting deoxyuracil (dU) containing nucleic acid molecules in the sample, a deoxynucleotide mix including dUTP, and a DNA polymerase to form a polymerase chain reaction mixture;

subjecting the polymerase chain reaction mixture to conditions suitable for digesting deoxyuracil (dU) containing nucleic acid molecules present in the polymerase chain reaction mixture, and one or more polymerase chain reaction cycles comprising a denaturation treatment, a hybridization treatment, and an extension treatment, thereby forming primary extension products comprising the target nucleotide sequence or a complement thereof;

blending the primary extension products with a ligase and one or more oligonucleotide probe sets to form a ligation reaction mixture, wherein each oligonucleotide probe set comprises (a) a first oligonucleotide probe having a 5' primer-specific portion and a 3' target nucleotide sequence-specific portion, and (b) a second oligonucleotide probe having a 5' target nucleotide sequence-specific portion and a 3' primer-specific portion, and wherein the first and second oligonucleotide probes of a probe set are configured to hybridize, in a base specific manner, on a complementary target nucleotide sequence of a primary extension product;

subjecting the ligation reaction mixture to one or more ligation reaction cycles whereby the first and second oligonucleotide probes of the one or more oligonucleotide probe sets are ligated together to form ligated product sequences in the ligation reaction mixture wherein each ligated product sequence comprises the 5' primer-specific portion, the target-specific portions, and the 3' primer-specific portion;

providing one or more secondary oligonucleotide primer sets, each secondary oligonucleotide primer set comprising (a) a first secondary oligonucleotide primer comprising the same nucleotide sequence as the 5' primer-specific portion of the ligated product sequence and (b) a second secondary oligonucleotide primer comprising a nucleotide sequence that is complementary to the 3' primer-specific portion of the ligated product sequence;

blending the ligated product sequences, the one or more secondary oligonucleotide primer sets, the one or more enzymes capable of digesting deoxyuracil (dU) containing nucleic acid molecules, a deoxynucleotide mix including dUTP, and a DNA polymerase to form a second polymerase chain reaction mixture;

subjecting the second polymerase chain reaction mixture to conditions suitable for digesting deoxyuracil (dU) containing nucleic acid molecules present in the second polymerase chain reaction mixture, and one or more polymerase chain reaction cycles comprising a denaturation treatment, a hybridization treatment, and an extension treatment thereby forming secondary extension products; and

detecting and distinguishing the secondary extension products in the sample to identify the presence of one or more nucleic acid molecules containing target nucleotide sequences differing from nucleotide sequences in other nucleic acid molecules in the sample by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues.

2. A method for identifying, in a sample, one or more nucleic acid molecules containing a target nucleotide sequence differing from nucleotide sequences in other nucleic acid molecules in the sample, or other samples, by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues, said method comprising:

providing a sample containing one or more nucleic acid molecules potentially containing the target nucleotide sequence differing from the nucleotide sequences in other nucleic acid molecules by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues;

providing one or more enzymes capable of digesting deoxyuracil (dU) containing nucleic acid molecules present in the sample;

providing one or more primary oligonucleotide primer sets, each primary oligonucleotide primer set comprising (a) a first primary oligonucleotide primer that comprises a nucleotide sequence that is complementary to a sequence adjacent to the target nucleotide sequence and (b) a second primary oligonucleotide primer that comprises a nucleotide sequence that is complementary to a portion of an extension product formed from the first primary oligonucleotide primer;

blending the sample, the one or more primary oligonucleotide primer sets, the one or more enzymes capable of digesting deoxyuracil (dU) containing nucleic acid molecules in the sample, a deoxynucleotide mix including dUTP, and a DNA polymerase to form a polymerase chain reaction mixture;

subjecting the polymerase chain reaction mixture to conditions suitable for digesting deoxyuracil (dU) containing nucleic acid molecules present in the polymerase chain reaction mixture, and one or more polymerase chain reaction cycles comprising a denaturation treatment, a hybridization treatment, and an extension treatment, thereby forming primary extension products comprising the target nucleotide sequence or a complement thereof;

blending the primary extension products with a ligase and one or more oligonucleotide probe sets to form a ligation reaction mixture, wherein each oligonucleotide probe set comprises (a) a first oligonucleotide probe having a 5' portion and a 3' target nucleotide sequence-specific portion, and (b) a second oligonucleotide probe having a 5' target nucleotide sequence-specific portion and a 3' portion, wherein the 5' portion of the first oligonucleotide probe of the probe set is complementary to a portion of the 3' portion of the second oligonucleotide probe, wherein one probe of the probe set comprises a detectable signal generating moiety, and wherein the first and second oligonucleotide probes of a probe set are configured to hybridize, in a base specific manner, on a complementary target nucleotide sequence of a primary extension product;

subjecting the ligation reaction mixture to one or more ligation reaction cycles whereby the first and second oligonucleotide probes of the one or more oligonucleotide probe sets are ligated together to form ligated product sequences in the ligation reaction mixture wherein each ligated product sequence comprises the 5' portion, the target-specific portions, the 3' portion, and the detectable signal generating moiety;

hybridizing the 5' portion of the ligated product sequence to its complementary 3' portion;

detecting signal from the detectable signal generating moiety that is produced upon said hybridizing; and

distinguishing ligated product sequences in the sample based on said detecting to identify the presence of one or more nucleic acid molecules containing target nucleotide sequences

differing from nucleotide sequences in other nucleic acid molecules in the sample by one or more nucleotides, one or more copy numbers, one or more transcript sequences, and/or one or more methylated residues.

3. The method of claim 1 further comprising:

contacting the sample with at least a first methylation sensitive enzyme to form a restriction enzyme reaction mixture prior to, or concurrent with, said blending to form a polymerase chain reaction mixture, wherein said first methylation sensitive enzyme cleaves nucleic acid molecules in the sample that contain one or more unmethylated residues within at least one methylation sensitive enzyme recognition sequence, and whereby said detecting involves detection of one or more nucleic acid molecules containing the target nucleotide sequence, wherein said nucleic acid molecules originally contained one or more methylated residues.

4. The method of claim 3, wherein the first primary

oligonucleotide primer comprises a nucleotide sequence that is complementary to a region of the target nucleotide sequence that is upstream of the one or more methylated residues and the second primary oligonucleotide primer comprises a nucleotide sequence that is the same as a region of the target nucleotide sequence that is downstream of the one or more methylated residues.

5. The method of claim 3 further comprising:

subjecting the restriction enzyme reaction mixture to a bisulfite treatment under conditions suitable to convert unmethylated cytosine residues to uracil residues prior to said blending to form a polymerase chain reaction mixture, wherein the first primary oligonucleotide primer of the provided primary oligonucleotide primer set comprises a nucleotide sequence that is complementary to the bisulfite-treated target nucleotide sequence containing the one or more methylated, uncleaved restriction sites and the second primary oligonucleotide primer of the provided primary oligonucleotide primer set comprises a nucleotide sequence that is complementary to a portion of the extension product formed from the first oligonucleotide primer.

6. The method of claim 5 further comprising:

providing one or more second methylation sensitive enzymes that cleave nucleic acid molecules containing unmethylated residues within a methylation sensitive enzyme recognition sequence; and

blending the at least one second methylation sensitive enzyme with the polymerase chain reaction mixture comprising the bisulfite-treated restriction enzyme reaction mixture to form a second restriction enzyme reaction mixture, wherein said second methylation sensitive enzyme

cleaves nucleic acid molecules potentially present in the sample that contain one or more unmethylated residues within at least one methylation sensitive enzyme recognition sequence during said hybridization treatment.

7. The method of claim 5, wherein one or both primary oligonucleotide primers of the primary oligonucleotide primer set have a 3' portion comprising a cleavable nucleotide or nucleotide analogue and a blocking group, such that the 3' end of said primer or primers is unsuitable for polymerase extension, said method further comprising:

cleaving the cleavable nucleotide or nucleotide analog of one or both oligonucleotide primers during said hybridization treatment, thereby liberating free 3'OH ends on one or both oligonucleotide primers prior to said extension treatment.

8. The method of claim 7, wherein the first primary oligonucleotide primer of the primary oligonucleotide primer set comprises a 5' portion having a nucleotide sequence that is the same as a nucleotide sequence portion in a bisulfite-treated unmethylated target sequence to which the primary oligonucleotide primer hybridizes to, but has one or more nucleotide sequence mismatches to a corresponding nucleotide sequence portion in the bisulfite-treated methylated target sequence.

9. The method of claim 8, wherein the DNA polymerase lacks 5' nuclease activity, 3' nuclease activity, and strand displacing activity.

10. The method of claim 4 further comprising:
providing one or more blocking oligonucleotides capable of hybridizing to a region of the bisulfite-treated target nucleotide sequence containing unmethylated residues; and contacting the polymerase chain reaction mixture comprising the bisulfite-treated restriction enzyme reaction mixture with said one or more blocking oligonucleotides prior to said subjecting, wherein said one or more blocking oligonucleotides hybridize to complementary bisulfite treated target nucleic acid sequences during said hybridization treatment and impede primary oligonucleotide primer extension during said extension treatment.

11. The method of claim 1, wherein one or both primary oligonucleotide primers of the primary oligonucleotide primer set have a 3' portion comprising a cleavable nucleotide or nucleotide analogue and a blocking group, such that the 3' end of said primer or primers is unsuitable for polymerase extension, said method further comprising:

cleaving the cleavable nucleotide or nucleotide analog of one or both oligonucleotide primers during said hybridization treatment, thereby liberating free 3'OH ends on one or both oligonucleotide primers prior to said extension treatment.

12. The method of claim 10, wherein the cleavable nucleotide comprises one or more RNA bases.

13. The method of claim 12, wherein the first primary oligonucleotide primer of the primary oligonucleotide primer set comprises a 5' portion having a nucleotide sequence that is the same as a nucleotide sequence portion in a wildtype nucleic acid molecule to which the primary oligonucleotide primer hybridizes to, but has one or more nucleotide sequence mismatches to a corresponding nucleotide sequence portion in the target nucleic acid molecule.

14. The method of claim 13, wherein the one or more sequence mismatches is located two nucleotide bases from the 5' end of the first primary oligonucleotide primer, and/or three nucleotide bases from the 5' end of the first primary oligonucleotide primer.

15. The method of claim 13, wherein the DNA polymerase lacks 5' nuclease activity, 3' nuclease activity, and strand displacing activity.

16. The method of claim 1, wherein the second oligonucleotide probe of the oligonucleotide probe set further comprises a unitaq detection portion, thereby forming ligated product sequences comprising the 5' primer-specific portion, the target-specific portions, the unitaq detection portion, and the 3' primer-specific portion, said method further comprising:

providing one or more unitaq detection probes, wherein each unitaq detection probe hybridizes to a complementary unitaq detection portion and said detection probe comprises a quencher molecule and a detectable label that are separated from each other;

adding the one or more unitaq detection probes to the second polymerase chain reaction mixture; and

hybridizing the one or more unitaq detection probes to complementary unitaq detection portions on the ligated product sequence or complement thereof during said subjecting the second polymerase chain reaction mixture to conditions suitable for one or more polymerase chain reaction cycles, whereby the quencher molecule and the detectable label are cleaved from the one or more unitaq detection probes during the extension treatment, whereby said detecting involves the detection of the cleaved detectable label.

17. The method of claim 1, said method further comprising:

providing one or more oligonucleotide detection probes, wherein each oligonucleotide detection probe hybridizes to a ligation product junction portion or its complement, and said detection probe comprises a quencher molecule and a detectable label that are separated from each other;

adding the one or more oligonucleotide detection probes to the second polymerase chain reaction mixture; and

hybridizing the one or more oligonucleotide detection probes to complementary detection portions on the ligated product sequence or complement thereof during said subjecting the second polymerase chain reaction mixture to conditions suitable for one or more polymerase chain reaction cycles, whereby the quencher molecule and the detectable label are cleaved from the one or more oligonucleotide detection probes during said extension treatment, whereby said detecting involves the detection of the cleaved detectable label.

18. The method of claim 2, said method further comprising:

immobilizing at least one strand of each primary extension product on a solid support prior to or subsequent to said ligating, wherein said ligated product sequence is hybridized to the immobilized primary extension product; and

removing unligated oligonucleotides probes and non-target specific ligation products from the sample after said ligating; and

denaturing the ligation product sequence from the immobilized primary extension product prior to said hybridizing.

19. The method of claim 1, wherein the 3' portion of the first oligonucleotide probe of the oligonucleotide probe set comprises a cleavable nucleotide or nucleotide analogue and a blocking group, such that the 3' end is unsuitable for polymerase extension or ligation, said method further comprising;

cleaving the cleavable nucleotide or nucleotide analog of the first oligonucleotide probe when said probe is hybridized to its complementary target nucleotide sequence of the primary extension product, thereby liberating a 3'OH on the first oligonucleotide probe prior to said ligating.

20. The method of claim 1, wherein the second oligonucleotide probe has, at its 5' end, an overlapping identical nucleotide with the 3' end of the first oligonucleotide probe, and, upon hybridization of the first and second oligonucleotide probes of a probe set at adjacent positions on a complementary target nucleotide sequence of a primary extension product to form a junction, the overlapping identical nucleotide of the second oligonucleotide probe forms a flap at the junction with the first oligonucleotide probe, said method further comprising:

cleaving the overlapping identical nucleotide of the second oligonucleotide probe with an enzyme having 5' nuclease activity thereby liberating a phosphate at the 5' end of the second oligonucleotide probe prior to said ligating.

21. The method of claim 1, wherein the one or more oligonucleotide probe sets further comprise a third oligonucleotide probe having a target-specific portion, wherein the second and third oligonucleotide probes of a probe set are configured to hybridize adjacent to one another on the target nucleotide sequence with a junction between them to allow ligation between the second and third oligonucleotide probes to form a ligated product sequence comprising the first, second, and third oligonucleotide probes of a probe set.

22. The method of claim 1, wherein the sample is selected from the group consisting of tissue, cells, serum, blood, plasma, amniotic fluid, sputum, urine, bodily fluids, bodily secretions, bodily excretions, cell-free circulating nucleic acids, cell-free circulating tumor nucleic acids, cell-free circulating fetal nucleic acids in pregnant woman, circulating tumor cells, tumor, tumor biopsy, and exosomes.

23. The method of claim 1, wherein the one or more target nucleotide sequences are low-abundance nucleic acid molecules comprising one or more nucleotide base mutations, insertions, deletions, translations, splice variants, miRNA variants, alternative transcripts, alternative start sites, alternative coding sequences, alternative non-coding sequences, alternative splicings, exon insertions, exon deletions, intron insertions, or other rearrangement at the genome level and/or methylated nucleotide bases.

24. The method of claim 23, wherein the low-abundance nucleic acid molecules with one or more nucleotide base mutations, insertions, deletions, translocations, splice variants, miRNA variants, alternative transcripts, alternative start sites, alternative coding sequences, alternative non-coding sequences, alternative splicings, exon insertions, exon deletions, intron insertions, or other rearrangement at the genome level, and/or methylated nucleotide bases are identified and distinguished from a high-abundance of nucleic acid molecules in the sample having a similar nucleotide sequence as the low abundance nucleic acid molecules but without the one or more nucleotide base mutations, insertions, deletions, translocations, splice variants, miRNA variants, alternative transcripts, alternative start sites, alternative coding sequences, alternative non-coding sequences, alternative splicings, exon insertions, exon deletions, intron insertions, or other rearrangement at the genome level, and/or methylated nucleotide bases.

25. The method of claim 24, wherein the copy number of one or more low-abundance target nucleotide sequences are quantified relative to the copy number of the high-abundance nucleic acid molecules in the sample.

26. The method of claim 1, wherein the one or more target nucleotide sequences are quantified or enumerated.

27. The method of claim 26, wherein the one or more target nucleotide sequences are quantified or enumerated relative to other nucleotide sequences in the sample or in another sample.

28. The method of claim 27, wherein the relative copy number of the one or more target nucleotide sequences are quantified or enumerated.

29. The method of claim 1 further comprising: diagnosing or prognosing a disease state based on said identifying.

30. The method of claim 1 further comprising: distinguishing a genotype or disease predisposition based on said identifying.