

CLAIMS

What is claimed is:

1. ~~—— A method for detecting and/or quantifying one or more target amplicons produced by amplification, wherein the detecting and/or quantifying is carried out during amplification or after an amplification endpoint has been reached, the method comprising:

preparing an amplification reaction mixture comprising:
—— sample nucleic acids;
—— at least one target-specific primer pair;
—— an optional probe, wherein at least one primer of the target-specific primer pair or the probe, if present, is labeled with a fluorescent dye; and
—— a fluorescent double-stranded DNA-binding dye, where fluorescence from the dye is capable of quenching fluorescent signal from the labeled primer or probe, if present;

subjecting the amplification mixture to amplification; and
detecting fluorescent signal to detect and/or quantify the one or more target amplicons.~~
12. A method for detecting and/or quantifying one or more target amplicons produced by amplification, wherein the detecting and/or quantifying is carried out during amplification or after an amplification endpoint has been reached, the method comprising:

preparing an amplification reaction mixture comprising:
sample nucleic acids;
at least one target-specific primer pair, wherein at least one primer in the target-specific primer pair comprises a nucleotide tag at the 5' end of the primer;

at least one fluorescently labeled primer or probe that is capable of annealing to the nucleotide tag, directly or via one or more intervening primers, whereby the label can become linked to the nucleotide tag; and

a fluorescent double-stranded DNA-binding dye, where
fluorescence from the double-stranded DNA-binding dye is capable of quenching
fluorescent signal from the labeled primer or probe;
subjecting the amplification mixture to amplification; and
detecting fluorescent signal to detect and/or quantify the one or more
target amplicons.

23. The method of claim 12, wherein the fluorescence from the double-stranded DNA-binding dye quenches fluorescent signal from the labeled primer or probe when the labeled primer or probe is incorporated into, or hybridized to, an amplification product.

34. A method for detecting an allele in a sample, the method comprising:
preparing an amplification mixture comprising:
sample nucleic acids;
two allele-specific primer pairs, wherein:
at least one primer in each primer pair is specific for
an allele and is tagged with a distinct nucleotide tag at the 5' end of the primer; and
the other primer in each pair can be the same or
different from one another;
at least two differently fluorescently labeled primers or
probes, each capable of annealing to one of the nucleotide tags, directly or via one or more
intervening primers, whereby one label can become linked to one nucleotide tag and a
different label can become linked to the other nucleotide tag;

a fluorescent double-stranded DNA-binding dye, where
fluorescence from the double-stranded DNA-binding dye is capable of quenching
fluorescent signal from the labeled primers or probes;
subjecting the amplification mixture to amplification; and
detecting fluorescent signal to detect the allele in the sample.

~~5. The method of claim 4, wherein the amplification mixture additionally comprises a fluorescent double-stranded DNA-binding dye, where fluorescence from the dye is capable of quenching fluorescent signal from the labeled primers or probes.~~

46. The method of claim 35, wherein the fluorescence from the double-stranded DNA-binding dye quenches fluorescent signal from the labeled primers or probes when the labeled primers or probes are incorporated into, or hybridized to, an amplification product.

57. A method for detecting an allele in a sample, the method comprising:
preparing an amplification mixture comprising:
sample nucleic acids;
two allele-specific primer pairs, wherein:
at least one primer in each primer pair is specific for
an allele and is tagged with a distinct nucleotide tag at the 5' end of the primer; and
the other primer in each pair can be the same or
different from one another;
at least two differently fluorescently labeled primers or
probes, each capable of annealing to one of the nucleotide tags, directly or via one or more
intervening primers, whereby one label can become linked to one nucleotide tag and a
different label can become linked to the other nucleotide tag;

~~The method of claim 4, wherein two differently labeled primers are employed, additionally comprising including in the reaction one or more quencher oligonucleotide(s) that comprise(s) a sequence that is capable of hybridizing to at least part of the nucleotide tag(s) and a fluorescence quencher, wherein hybridization to unincorporated fluorescently labeled primer(s) quenches the fluorescent label(s);-~~

subjecting the amplification mixture to amplification; and
detecting fluorescent signal to detect the allele in the sample.

68. The method of claim 57, wherein the fluorescence quencher is at the 3' end of the quencher oligonucleotide.

79. The method of claim 57, wherein the fluorescence quencher is attached to an internal nucleotide of the quencher oligonucleotide.

810. The method of claim 57, wherein the amplification mixture comprises at least two quencher oligonucleotides, one specific for each nucleotide tag.

~~911.~~ A method for detecting an allele in a sample, the method comprising:

preparing an amplification mixture comprising:

sample nucleic acids;

two allele-specific oligonucleotides, wherein each oligonucleotide comprises a target-specific sequence linked to a distinct 3' nucleotide tag; and

at least two differently fluorescently labeled primers or probes, each capable of annealing to one of the nucleotide tags, whereby one label can become linked to one nucleotide tag and a different label can become linked to the other nucleotide tag;

one or more quencher oligonucleotide(s) that comprise(s) a sequence that is capable of hybridizing to at least part of the nucleotide tag(s) and a fluorescence quencher, wherein hybridization to unincorporated fluorescently labeled primer(s) quenches the fluorescent label(s);

subjecting the amplification mixture to amplification; and

detecting fluorescent signal to detect the allele in the sample.

~~12. The method of claim 11, wherein two differently labeled primers are employed, additionally comprising including in the reaction one or more quencher oligonucleotide(s) that comprise(s) a sequence that is capable of hybridizing to at least part of the nucleotide tag(s) and a fluorescence quencher, wherein hybridization to unincorporated fluorescently labeled primer(s) quenches the fluorescent label(s).~~

~~1013.~~ The method of claim ~~912~~, wherein the fluorescence quencher is at the 3' end of the quencher oligonucleotide.

~~1114.~~ The method of claim ~~912~~, wherein the fluorescence quencher is attached to an internal nucleotide of the quencher oligonucleotide.

~~1215.~~ The method of claim ~~912~~, wherein the amplification mixture comprises at least two quencher oligonucleotides, one specific for each nucleotide tag.

~~16. A method for adding nucleotide sequences to one or more target nucleic acids by amplification, the method comprising:~~

preparing an amplification mixture for each target nucleic acid, said amplification mixture comprising:

- sample nucleic acids;
- an inner forward primer comprising a target specific sequence and a first nucleotide tag at the 5' end of the primer;
- an inner reverse primer comprising a target specific sequence and a second nucleotide tag at the 5' end of the primer;
- an outer forward primer comprising the first nucleotide tag;

and

- an outer reverse primer comprising the second nucleotide tag, wherein one or both outer primers can, optionally, include one or more additional nucleotide sequences to be added to the target nucleic acid; and

subjecting each amplification mixture to amplification to produce a plurality of target amplicons comprising tagged target nucleotide sequences, each comprising first and second nucleotide tags linked to the target nucleotide sequence.

17. — A method for tagging a plurality of target nucleic acids in a sample with common nucleotide tags, the method comprising:

- contacting the sample with:
 - a plurality of 5' oligonucleotides, one for each target nucleic acid, wherein each 5' oligonucleotide comprises a first nucleotide tag that is linked, to and 5' of, a target specific sequence;

- a plurality of 3' oligonucleotides, one for each target nucleic acid, wherein each 3' oligonucleotide comprises a target specific sequence that is linked to, and 5' of, a second nucleotide tag;

- wherein the target specific sequence of each 5' oligonucleotide hybridizes to a target nucleic acid immediately adjacent to the target specific sequence of the 3' oligonucleotide, with an overlap such that one or more of the 5' most base(s) of the 3' oligonucleotide is/are displaced from the target nucleic acids, forming a flap;

- a flap endonuclease; and
 - a ligase;
 - to produce a plurality of tagged target nucleic acids, each comprising the first and second tags.

18.—~~The method of claim 17, additionally comprising removing unligated oligonucleotides and amplifying the tagged target nucleic acids using primers specific for the first and second nucleotide tags.~~

19.—~~A method for determining the methylation state of cytosine in a target nucleic acid sequence in a sample, the method comprising:~~

~~treating the sample to convert methylated cytosine(s) to uracil(s) in the target nucleic acids to produce a treated sample;~~

~~contacting the treated sample with:~~

~~—— a first 5' oligonucleotide comprising a first nucleotide tag that is linked to, and 5' of, a first melting temperature discriminator sequence that is linked to, and 5' of, a 5' target-specific sequence, wherein the 3' most base is a G;~~

~~—— a first 3' oligonucleotide comprising a G linked to a 3' target-specific sequence,~~

~~—— wherein the target-specific sequence of the first 5' oligonucleotide hybridizes to a target nucleic acid immediately adjacent to the target-specific sequence of the first 3' oligonucleotide, with an overlap such that at least the G of the 3' oligonucleotide is displaced from the target nucleic acids, forming a flap;~~

~~—— a second 5' oligonucleotide comprising the same first nucleotide tag that is linked to, and 5' of, a second melting temperature discriminator sequence that is linked to, and 5' of, a 5' target-specific sequence, wherein the 3' most base is an A;~~

~~—— a second 3' oligonucleotide comprising an A linked to the 3' target-specific sequence;~~

~~—— wherein the target-specific sequence of the second 5' oligonucleotide hybridizes to a target nucleic acid immediately adjacent to the target-specific sequence of the second 3' oligonucleotide, with an overlap such that at least the A of the 3' oligonucleotide is displaced from the target nucleic acids, forming a flap;~~

~~—— a flap endonuclease; and~~

~~—— a ligase~~

~~to produce a ligation product from the first 5' and 3' oligonucleotides if the target nucleic acid comprised a methylated cytosine or from the second 5' and 3' oligonucleotides if the target nucleic acids comprised an unmethylated cytosine.~~

20.—~~The method of claim 19, additionally comprising removing unligated oligonucleotides and amplifying the tagged target nucleic acids using a forward primer specific for the first nucleotide tag and a reverse primer that is specific for a target nucleotide sequence in the ligation product.~~

21.—~~The method of claim 28, additionally comprising determining which ligation product was produced by melting curve analysis.~~

22.—~~A method for detecting a relative copy number difference in target nucleic acids in a sample, the method comprising:~~

~~subjecting a sample to preamplification using primers capable of amplifying a plurality of target nucleic acids to produce a plurality of target amplicons, so that the relative copy numbers of the target nucleic acids is substantially maintained, where some of the target nucleic acids are present on first chromosome and some of the target nucleic acids are present on a second, different chromosome;~~

~~determining the number of copies of target amplicons derived from the first chromosome and the number of copies of target amplicons derived from the second chromosome;~~

~~and determining the relative copy difference for the first and second chromosomes, wherein said method can detect a relative copy number difference less than 1.5.~~

23.—~~The method of claim 22, wherein the plurality of target nucleic acids comprises at least about 10 target sequences on each chromosome.~~

24.—~~The method of claim 23, wherein the plurality of target nucleic acids comprises at least about 100 target sequences on each chromosome.~~

25.—~~The method of claims 24-24, wherein the number of copies of target amplicons derived from the first chromosome and the number of copies of target amplicons derived from the second chromosome are determined by a method comprising amplification.~~

26.—~~The method of claim 25, wherein the amplification comprises digital amplification.~~

~~27.—The method of claims 22-24, wherein the number of copies of target amplicons derived from the first chromosome and the number of copies of target amplicons derived from the second chromosome are determined by a method comprising DNA sequencing.~~

~~28.—A method for detecting a relative copy number difference between alleles at one or more target loci in a sample comprising a first allele and a second, different allele at at least one target locus, the method comprising:~~

~~subjecting a sample to preamplification using primers capable of amplifying the first and second alleles to produce a plurality of target amplicons, so that the relative copy numbers of the first and second alleles is substantially maintained;~~

~~distributing the target amplicons into a plurality of amplification mixtures and carrying out digital amplification;~~

~~determining the number of amplification mixtures that contain a target amplicon derived from the first allele, and determining the number of amplification mixtures that contain a target amplicon derived from the second allele;~~

~~determining the ratio of amplification mixtures that contain the first allele to those that contain the second allele to detect the relative copy difference for the first and second alleles, wherein said method can detect a relative copy number difference less than 1.5.~~

~~29.—The method of claim 22-28, wherein the relative copy number difference is at least 1.02.~~

~~30.—The method of claims 22-29, wherein preamplification is carried out for between 2 and 25 cycles.~~

~~31.—The method of claim 31, wherein preamplification is carried out for between 5 and 20 cycles.~~

~~32.—The method of claims 22-31, wherein at least one primer of each primer pair employed for preamplification comprises a nucleotide tag.~~

~~33.—The method of claim 32, wherein the nucleotide tag comprises a universal tag.~~

34. — The method of claim 32, wherein the nucleotide tag comprises a chromosome-specific nucleotide tag;

35. — A method for detecting fetal aneuploidy in a maternal bodily fluid sample from a pregnant subject, the method comprising:

subjecting a sample of a maternal bodily fluid sample, or a fraction thereof, to preamplification using primer pairs capable of amplifying at least a plurality of target nucleic acids to produce a plurality of target amplicons, so that the relative copy numbers of the target nucleic acids is substantially maintained, where some of the target nucleic acids are present on a first chromosome and some of the target nucleic acids are present on a second, different chromosome, wherein:

—— each primer employed for preamplification comprises a nucleotide tag, so that preamplification produces target amplicons comprising first a first nucleotide tag at one end and a second nucleotide tag at the other end, wherein all target amplicons derived from a given chromosome comprise the same first and second nucleotide tags; and

—— all target amplicons derived from a given chromosome are detectable with a common probe;

distributing the target amplicons into a plurality of amplification mixtures and carrying out multiplex digital amplification using:

—— a primer pair specific for the first and second nucleotide tags in target amplicons derived from the first chromosome;

—— a common probe specific for the target amplicons derived from the first chromosome;

—— a primer pair specific for the first and second nucleotide tags in target amplicons derived from the second chromosome; and

—— a common probe specific for the target amplicons derived from the second chromosome;

determining the number of amplification mixtures that contain a target amplicon derived from the first chromosome, and determining the number of amplification mixtures that contain a target amplicon derived from the second chromosome;

determining the ratio of amplification mixtures that contain the first chromosome to those that contain the second to detect the relative copy difference for the first and second alleles.

36. — The method of claim 35, where each common probe detects a chromosome-specific motif.

37. — The method of claim 35, wherein the probes are labeled with different fluorescent labels.

38. — The method of claims 35 or 37, wherein the plurality of target nucleic acids comprises at least about 10 target sequences on each chromosome.

39. — The method of claim 38, wherein the plurality of target nucleic acids comprises at least about 100 target sequences on each chromosome.

40. — The method of claims 35-39, wherein the relative copy number difference is at least 1.02.

41. — The method of claims 35-40, wherein preamplification is carried out for between 2 and 25 cycles.

42. — The method of claim 41, wherein preamplification is carried out for between 5 and 20 cycles.

43. — A method for detecting a relative copy number difference between at least two loci in genomic DNA or RNA in a sample, the method comprising:

quantifying the amount, in the sample, of a first non-coding RNA expressed from a chromosomal region linked to a first locus;

quantifying the amount, in the sample, of a second non-coding RNA expressed from a chromosomal region linked to a second locus;

determining a ratio of the amount of the first non-coding RNA to the amount of the second non-coding RNA, wherein a ratio significantly different from one indicates a copy number difference between the first and second locus.

44. — The method of claim 43, wherein the first and second non-coding RNAs comprise first and second single-stranded, non-coding RNAs.

45.—The method of claim 43, wherein the first and second non-coding RNAs comprise first and second double-stranded, non-coding RNAs.

46.—The method of claim 43, wherein the first and second non-coding RNAs comprise first and second miRNAs.

47.—A method for detecting a relative copy number difference between at least two loci in genomic DNA a sample, the method comprising:

producing, from the sample, a first DNA sequencing template that comprises, 5' to 3', a primer binding site for a forward DNA sequencing primer, linked directly, or via an intervening sequence, to a first target nucleotide sequence derived from the first locus, which is linked directly, or via an intervening sequence, to a primer binding site for a reverse DNA sequencing primer;

producing, from the sample, a second DNA sequencing template that comprises, 5' to 3', the primer binding site for the forward DNA sequencing primer, linked directly, or via an intervening sequence, to a second target nucleotide sequence derived from the second locus, which is linked directly, or via an intervening sequence, to a primer binding site for the reverse DNA sequencing primer, wherein:

the forward and reverse DNA sequencing primer binding sites are the same in both DNA sequencing templates; and

the first and second DNA sequencing templates are produced from the sample substantially in proportion to the copy number of the first and second loci in the sample;

determining the nucleotide sequences of the DNA sequencing templates;

quantifying the amount of first and second DNA sequencing templates;

determining a ratio of the amount of the first DNA sequencing template to the amount of the second DNA sequencing template to determine a copy number difference between the first and second locus.

48.—The method of claim 47, wherein the first and second DNA sequencing primers additionally comprise a barcode nucleotide sequence between the primer binding site for the forward DNA sequencing primer and the first and second target nucleotide sequences, respectively.

~~49.—The method of claim 47, wherein the first and second DNA sequencing primers additionally comprise a barcode nucleotide sequence between the first and second target nucleotide sequences, respectively, and the primer binding site for the forward DNA sequencing primer.~~

~~50.—The method of any of the preceding claims, wherein the amplification mixtures are formed in, or distributed into, separate compartments of a microfluidic device prior to amplification.~~

~~51.—The method of claim 50, wherein the microfluidic device is fabricated, at least in part, from an elastomeric material.~~

~~52.—The method of claims 1, 4, 11, 16, 17, 19, 22, 28, 43, or 47, wherein the sample comprises a sample of a maternal bodily fluid, or a fraction thereof, from a pregnant subject.~~

~~53.—The method of claims 1, 4, 11, 16, 17, 19, 22, 28, 43, or 47, wherein at least some of the target amplicons, alleles, target nucleic acids, or loci are derived from, or comprise fetal, DNA.~~

~~54.—The method of claim 53, wherein the sample comprises a sample of maternal blood, or a fraction thereof, and at least some of the target nucleic acids comprise fetal DNA.~~

~~55.—A method for detecting and/or quantifying one or more fetal target nucleic acids in a maternal bodily fluid sample from a pregnant subject, the method comprising:~~

~~treating the sample to enrich for amplifiable fetal nucleic acids and produce a treated sample, wherein the treated sample comprises a higher percentage of fetal nucleic acids that are capable of being amplified, as compared to the percentage of maternal nucleic acids that are capable of being amplified;~~

~~amplifying the one or more fetal target nucleic acids; and
detecting and/or quantifying the one or more fetal target nucleic acids.~~

~~56.—The method of claim 55, wherein a maternal bodily fluid is treated to enrich for amplifiable fetal DNA without prior fractionation.~~

~~57.—The method of claims 35, 52, 55, and 56, wherein the maternal bodily fluid is selected from the group consisting of whole blood, plasma, urine, and cervico-vaginal secretions.~~

~~58.—The method of any of claims 55-57, wherein said treating comprises enriching the sample for short nucleic acids.~~

~~59.—The method of claims 58 or 62, wherein said treating comprises physical enrichment based on size.~~

~~60.—The method of claim 59, wherein nucleic acids from a maternal bodily fluid sample are fractionated based on nucleic acid size, and the fractions are assayed to determine which fraction(s) comprise(s) short nucleic acids.~~

~~61.—The method of claim 60, wherein the method comprised determining whether two target nucleic acid sequences that are more than about 300 nucleic acids apart in the genome are found together on individual nucleic acids or are found on separate nucleic acids.~~

~~62.—The method of claim 58 or 60, wherein said treating comprises enriching the sample for nucleic acids that are about 300 nucleic acids or less in length.~~

~~63.—The method of claims 58 or 62, wherein said treating comprises selective amplification based on size.~~

~~64.—The method of claims 55-63, wherein the method comprises determining a fetal genotype.~~

~~65.—The method of claims 55-63, wherein the method comprises detecting the presence of a mutation or fetal aneuploidy.~~