WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 06/45281

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V.2 - Citations and explanations

As to claim 16-17, Findlay teaches the method of claim 1, wherein the determination of the target's genetic data is used for the purpose of embryo selection in the context of in-vitro fertilization and wherein the determination of the target's genetic data is used for the purpose of prenatal genetic diagnosis (see pg 19, ln 15-30).

As to claim 19-23, Findlay teaches the determination of the target's genetic data is used for the purpose of making phenotypic predictions (sex diagnosis, trisomy diagnosis), and
where the likelihood of displaying some or all of the phenotypes is affected by other previously known phenotypic information; and wherein the determination of the target's genetic data is used for the purpose of making phenotypic predictions;
where the predictions are made by comparing the target's genetic data to known genetic markers found in the public domain;
wherein the determination of the target's genetic data is used for the purpose of making clinical decisions;
wherein the determination of the target's genetic data is used, in combination with phenotypic markers for the purpose of making clinical decisions;
wherein the determination of the target's genetic data is used for the purpose of screening for susceptibly to one or more diseases, where no family history of the disease is present (see pg 19, ln 16-30).

As to claim 24, Findlay teaches the determination of the target's genetic data is used for the purpose of screening for susceptibility to one or more phenotypes, where some or all of the phenotypes are multigenic (trialielic) (see pg 8, ln 1-2).

As to claim 25, Findlay teaches the knowledge of the target's genetic data is known to or suspected to contain spurious data from contaminating DNA or RNA (semen from multiple sources are present) (see pg 18, ln 29 - pg 19, ln 7).

As to claims 30 and 42, Findlay teaches a system and method wherein the measurement of multiple loci on a given segment of a given chromosome of a target individual is used to determine the number of instances of the given segment in the genome of the target individual, the method comprising:
(i) creating a set of one or more hypotheses about the number of instances of the given segment present in the genome of the target individual,
(ii) measuring the amount of genetic data for some of all of the possible alleles (number of alleles)at a plurality of loci (number of polymorphic loci) on the given segment,
(iii) determining the relative probability of each of the hypotheses given the measurements of the target individual's genetic data and possibly also the related individual's genetic data, and
(iv) using the relative probability (frequency distribution in a population) associated with each hypothesis to determine the most likely state of the actual genetic material of the target individual (see pg 22, ln 20-26).

As to claim 31, Findlay teaches the method of claim 30, where the determination of the number of instances of segments of chromosomes that are present in the target's genome is done in the context of screening for trisomy (see Fig 14 and pg 7, ln 16-17).

As to claim 36, Findlay teaches the method of claim30, where the target individual is a human embryo (see pg 9, ln 1-2).

As to claim 37, Findlay teaches the method of claim 30, wherein the target individual's genetic data has been amplified using Polymerase Chain Reaction (PCR) (see pg 9, ln 6-8).

As to claim 38, Findlay teaches the method of claim 30, wherein the target individual's genetic data has been measured using florescent in-situ hybridization (FISH), and combinations thereof (see pg 17, ln 1-2).

As to claim 39, Findlay teaches the method of claim 30, wherein the target individual's genetic data has been measured by analyzing substances taken from the extra-cellular genetic material found in maternal blood, cells (see pg 36, ln 26).

As to claims 40-41, Findlay teaches the method of claim 30,
wherein the determination of the number of chromosomes or chromosome segments in the target is used for the purpose of embryo selection in the context of in-vitro fertilization (preimplantation genetic diagnosis);
wherein the determination of the number of chromosomes or chromosome segments of the target is used for the purpose of prenatal genetic diagnosis (see pg 19, ln 15-30).

As to claim 44, Findlay teaches a method for determining the genetic data for a target individual, and also the number of instances of chromosomes or segments of chromosomes that are present in the target's genome,base on the imperfect knowledge of the target individual's genetic data, and knowledge of the genetic data of one or more individuals genetically related to the target (see pg 14, ln 1 - pg 16, ln 27), the method comprising:
(i) creating a set of one or more hypotheses about which segments of which chromosomes from the related individuals correspond to those segments found in the target individual's genome (appropriate chromosome) (see pg 8, ln 16-19),
(ii) creating a set of one or more hypotheses about the number of a given chromosomal segment present in the genome of the target,
(iii) measuring the amount of genetic data for each of the possible alleles at a plurality of loci on the given segment,
(iv) determining the relative probability of each of the hypotheses (genetic identification, human embryo identification, forensics, genetic identification by DNA fingerprinting) given the measurements of the target individual's genetic data and of the related individual's genetic data (see pg 9, ln 1-4 and pg 12, ln 26-28); and
(v) using the relative probabilities associated with each hypothesis to determine the most likely state (heterozygosity index, genetic marker selection)of the actual genetic material of the target individual (see pg 7, ln 25 - pg 8, ln 4).

(See Supplemental Box - Reasoned Statement)

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 06/45281

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V Citations and explanations

Claims 45-61 lack novelty under PCT Article 33(2) as being anticipated by US 2004/0137470 A1 (Dhallan).

As to claims 45 and 60, Dhallan teaches a system and method for making predictions regarding an individual, comprising:
(i) constructing models based on contingency tables built from publicly available information about gene-disease associations (the sequence of multiple loci of interest that cause or presuppose to a disease phenotype can be determined) (para [0591]); and (ii) applying models to make the predictions by operating on data pertaining to the individual (the number of mutant cells in the sample is used to determine patient prognosis, the degree and severity of the disease) (see para [0879]).

As to claims 46-48, Dhallan teaches an accuracy of the contingency tables that employ multiple independent variables can be refined using outcome data where only a subset of those independent variables was measured (the sequence of multiple loci of interest that cause or predispose to a disease phenotype can be determined) (see para [0591]);
wherein an accuracy of the contingency tables that employ multiple independent variables can be refined using data about the association of the independent variables (wild type DNA sequence and mutant DNA sequence) (see para [0879]);
wherein an accuracy of the contingency tables that employ multiple independent variable (independently varying) can be refined using data about the frequency of occurrence of certain values of the independent variables (see para [2645]).

As to claims 50, 52, 54, 56 and 58, Dhallan teaches the type of data pertaining to the individual comprises data taken from a group consisting of the individual's genotypic data (the number of mutant cells in the sample), the individual's phenotypic data, the individual's clinical data, and the individual's laboratory data (see para [0879]);
where the type of data also consists of data of a pathogen infecting the individual (detect an allele from a non-pathogenic bacterial strain amongst alleles from a pathogenic bacterial strain) (see para [0350]);
where said predictions concern topics selected from the group consisting of the individual's phenotypes, phenotype susceptibilities, possible clinical outcomes, lifestyle decisions, physical exercise, dietary habits, hormonal supplements, nutritional supplements, treatments for a disease, treatments for a pathogen, treatments for an undesirable condition, treatments with pharmaceuticals, and combinations thereof (see para [0828]);
where said predictions are used to generate a report for the individual or for an agent of the individual (see para [0226]); and
wherein the act of operating comprises operating on new data to update the individual's predictions where the data is taken from a group comprising new research data or new aggregated data on other subjects (loci of interest can be combined and treated as a single sample) (see para [0591]).

As to claims 49 and 61 Dhallan teaches a system and method for making predictions (detection of genetic disorders) regarding a first individual, comprising:
(i) creating and testing a plurality of models and using aggregated data from a second set of individuals for whom the outcome to be predicted is known;
(ii) calculating the relative accuracies of the various models for making the prediction given the data available to the first individual (quantitating a ratio for the alleles at a locus of interest) ; and
(iii) using the model that is identified as the most accurate to make a prediction for the first individual (see Abstract).

As to claims 51, 53, 55, 57 and 59, Dhallan teaches where the type data pertaining to the individual comprises data taken from a group consisting of the individual's genotypic data, the individual's phenotypic data, and the individual's clinical data, and the individual's laboratory data;
where the type of data also consists of data of a pathogen infecting the individual (see para [0350]);
where said predictions concern topics selected from the group consisting of the individual's phenotypes, phenotype susceptibilities, possible clinical outcomes, lifestyle decisions, physical exercise, mental exercise, dietary habits, hormonal supplements, nutritional supplements, treatments for a disease, treatments for a pathogen, treatments for an undesirable condition, treatments with pharmaceuticals, and combinations thereof (see para [0006]-[0029]);
where said predictions are used to generate a report for the individual or for an agent of the individual (see para [2260]);
wherein the act of operating comprises operating on new data to update the individual's predictions where the data is taken from a group comprising new research data or new aggregated data on other subjects (see table XV, Diagnostic Applications).

Claims 5 and 26-27 lack an inventive step under PCT Article 33(3) as being obvious over Findlay in view of Dhallan.

As to claims 5 and 26-27, Findlay teaches a confidence (prenatal diagnosis) is computed for each of the individual cells in the the cleaned embryonic genetic data (cells isolated from amniotic fluid) (see pg 14, ln 1-9 and pg 22, ln 4-28). Findlay does not specifically teach the prenatal diagnosis cells are SNP cells, or that the genetic data of one or more of the individuals include a plurality of SNPs. Dhallan teaches
fetal chromosomal abnormalities are determined by analyzing SNPs (see para [2693]);
the genetic data of one or more of the individuals includes the allele calls for a plurality of SNPs and the confidence with which each SNP is known (see fig 3A-3C and para [0101]); and
wherein the confidence in the SNP calls of the target individual is determined by computing the odds ratio of the probabilities that the SNP is correctly versus incorrectly called (SNP sites analyzed on chromosome 13 are present in the expected 50:50 ratio) (see Fig 12 and para [0117]).
It would have been obvious to one of ordinary skill in the art to combine the gene marker and amplification process as taught by Findlay with the methods for detection of genetic disorders as taught by Dhallan, because Dhallan teaches clinical techniques and practical applications to use a gene marker and amplification system, such as the system taught by Findlay.

(See Supplemental Box)

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Exhibit 1003 Page 1048

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 06/45261

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V Citations and explanations

Claims 15, 18, 29, 32-35 and 43 lack an inventive step under PCT Article 33(3) as being obvious over Findlay in view of US 2004/0236518 A1 to Pavlovic et al. (hereinafter 'Pavlovic').

As to claim 15, 18, 29, 32-35 and 43, Findlay teaches a method for determining the genetic data of a target individual. Findlay does not specifically teach the method as part of a computer algorithm, where the method comprises a hidden Markov model, making predictions using statistical models or using matched filtering.
Pavlovic teaches the computer algorithm comprises a hidden Markov model (see Fig 4, 5 and para [0060]); wherein the determination of the target's genetic data is used for the purpose of making predictions of phenotype susceptibility using statistical models and/or expert rules (combination of experts) (see para [013]);
including a computer implemented system configured to accomplish the method of claim 1 (see Fig 1A, 1B and para [0036]-[0037]);
where the determination of the relative probability of each hypothesis is made using the concept of matched filtering and where the determination of the relative probability of each hypothesis is made using quantitative techniques (probability equation) that do not make allele calls and where the mean and standard deviation of the measurement of each locus is either known, unknown, or uniform (see table 7 and para [0031]);
where the determination of the relative probability of each hypothesis is made using qualitative techniques that make use of allele calls; or where the determination of the relative probability of each hypothesis is made by making use of known alleles of reference sequence,and quantitative allele measurements (see para [0042]-[0044]); and a computer-implemented system configured to accomplish the method of claim 30 (see Fig 1A, 1B and para [0036]-[0037]).
It would have been obvious for one of ordinary skill in the art to combine the method for determining the genetic data of an individual as taught by Findlay with the expert system for gene prediction as taught by Pavlovic because a computer modeling and statistical analysis system, such as that taught by Pavlovic would be a required element in a system that fully utilized the genetic data identification system taught by Findlay.

Claims 1-61 have industrial applicability under PCT Article 33(4) as the subject matter claimed can be made or used in industry.

NeoGenomics
Exhibit 1003 Page 1049

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 06/45281

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8): G06F 19/00 (2007.01)
USPC: 702/20
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 702/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 702/1, 19; 705/2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Databases Searched: PubWEST (USPT,PGPB,EPAB,JPAB
Search Terms Used: DNA fingerprinting, sparse data, Markov, genetically related, haplotype, genome, alleles, relationship

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO 03/031646 A1 (FINDLAY et al) 17 April 2003 (07.04.2003), entire document, especially Fig 4-7, pg 6, ln 8 - pg 36, ln 26, table 6a, 6b	1-4, 6-14, 16-17, 19-25, 28, 30-31, 36-42, 44 ---------- 5, 15, 18, 26-27, 29, 32-35, 43
X ---- Y	US 2004/0137470 A1 (DHALLAN) 15 July 2004 (15.07.2004), entire document, especially Abstract, Fig 3A-3C, 12, para [0006] - [2693]	45-61 ---------- 5, 26-27
Y	US 2004/0236518 A1 (PAVLOVIC et al.) 25 November 2004 (25.11.2004), entire document, especially Fig 4, 5 and Fig 1A-1B, para [0036] - [0060	15, 18, 29, 32-35, 43

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2007 (02.05.2007)	28 SEP 2007
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2005)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 35506-501-061	**FOR FURTHER ACTION** See item 4 below	
International application No. PCT/US2006/045281	International filing date *(day/month/year)* 22 November 2006 (22.11.2006)	Priority date *(day/month/year)* 26 November 2005 (26.11.2005)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant GENE SECURITY NETWORK LLC		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

   - ☒ Box No. I — Basis of the report
   - ☐ Box No. II — Priority
   - ☐ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV — Lack of unity of invention
   - ☒ Box No. V — Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☐ Box No. VI — Certain documents cited
   - ☐ Box No. VII — Certain defects in the international application
   - ☐ Box No. VIII — Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 27 May 2008 (27.05.2008)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Nora Lindner e-mail: pt11.pct@wipo.int

Form PCT/IB/373 (January 2004)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/003547

A. CLASSIFICATION OF SUBJECT MATTER
INV. G06F19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2007/062164 A (GENE SECURITY NETWORK LLC [US]; RABINOWITZ MATTHEW [US]; BANJEVIC MILE) 31 May 2007 (2007-05-31) the whole document -/--	1-6,8-16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 2009	15/04/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Swarén, Peter

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/003547

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLELLA STEFANO ET AL: "QuantiSNP: an Objective Bayes Hidden-Markov Model to detect and accurately map copy number variation using SNP genotyping data." NUCLEIC ACIDS RESEARCH 2007, vol. 35, no. 6, 6 March 2007 (2007-03-06), pages 2013-2025, XP002506459 ISSN: 1362-4962	1-6,8-16
A	abstract page 2014, left-hand column, last paragraph - page 2017, left-hand column, last paragraph page 2018, left-hand column, last paragraph - page 2019, right-hand column, paragraph 1 page 2023, left-hand column, paragraph 1	17-33
A	FREEMAN JENNIFER L ET AL: "Copy number variation: new insights in genome diversity." GENOME RESEARCH AUG 2006, vol. 16, no. 8, August 2006 (2006-08), pages 949-961, XP002506460 ISSN: 1088-9051 figure 5	1,2,4-6, 8-16
X	US 2006/134662 A1 (PRATT MARK R [US] ET AL) 22 June 2006 (2006-06-22) abstract; claims 1-4; figures 1,2,5,6 paragraphs [0017], [0022], [0023], [0075], [0078], [0081] - [0092]	17-33
A	BEAUMONT MARK A ET AL: "The Bayesian revolution in genetics." NATURE REVIEWS. GENETICS APR 2004, vol. 5, no. 4, April 2004 (2004-04), pages 251-261, XP002521825 ISSN: 1471-0056 the whole document	1-6,8-33
A	OGINO SHUJI ET AL: "Bayesian analysis and risk assessment in genetic counseling and testing." THE JOURNAL OF MOLECULAR DIAGNOSTICS : JMD FEB 2004, vol. 6, no. 1, February 2004 (2004-02), pages 1-9, XP002521826 ISSN: 1525-1578 the whole document	1-6,8-33

NeoGenomics
Exhibit 1003 Page 1053

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2008/003547

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

1-6,8-33

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 4-6 and 8-16

implementation details of a method for determining the number of copies of a segment of a chromosome in the genome of a target individual by measurement of genetic data at a plurality of loci on the chromosome segment for the purpose of screening for a chromosomal condition in the context of prenatal genetic diagnosis during in-vitro fertilization embryo selection;

---

2. claims: 17-33

an allele calling method;

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3. claim: 3

probability determination of genetic measurements by integration of the probabilities over the possible set of parameters that vary across the measurement experiment;

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4. claim: 7

amplification of genetic material.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/003547

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007062164 A	31-05-2007	AU 2006318425 A1	31-05-2007
		CA 2632230 A1	31-05-2007
		EP 1960929 A2	27-08-2008
US 2006134662 A1	22-06-2006	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/34506

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/48; C12Q 1/68 (2009.01)
USPC - 702/20 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 33/48; C12Q 1/68 (2009.01)
USPC: 702/20 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Amer. Journal of Human Genetics, Vol 73

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,EPAB,JPAB): amplification, genotyping, fraction, locus specfic primer, ploidy, lysed, haplotype
esp@cenet: gene security, rabinowitz, johnson, locus specific, haplotype, genotyping, ploidy
Google Scholar: amplification, whole genome, multiple displacement, locus specific primers reconstruct haplotype geno

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/0259100 A1 (GUNDERSON et al.) 23 December 2004 (23.12.2004), abstract; para [0051], [0117], [0138], [0164], [0282].	1-3
Y	US 2003/0228613 A1 (BORNARTH et al.) 11 December 2003 (11.12.2003), abstract; para [0012], [0033].	1-3
Y	US 2006/0040300 A1 (DAPPRICH et al.) 23 February 2006 (23.02.2006), para [0030], [0057], [0064], [0111], [0191], [0198], [0203], [0204].	10-12, 20-22
Y	STEPHENS et al., A Comparison of Bayesian Methods for Haplotype Reconstruction from Population Genotype Data. Am J Human Genetics, November 2003, Vol 73, No 5, pages 1162-1169. especially page 1162, abstract.	10-12
Y	US 2005/0272073 A1 (VAISBERG et al.) 8 December 2005 (08.12.2005), abstract; para [0006], [0008], [0030], [0047], [0053].	20-22

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2009 (30.06.2009)	08 JUL 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Fo (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/34506

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-9, 13-19, 23-32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

NeoGenomics
Exhibit 1003 Page 1058

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/45335

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61B 17/435 (2009.01)
USPC - 600/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 600/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 600/33, 34, 435/325
(text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed
Search terms: preimplantation genetic diagnosis, IVF, in vitro fertilization, embryo, diagnose, probability, ploidy, characteristic, subcharacteristic, single cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILTON. Preimplantation genetic diagnosis and chromosome analysis of blastomeres using comparative genomic hybridization. Hum. Reprod. Update. January-February 2005 (01-02.2005), Vol. 11, No. 1, pages 33-41; Fig. 2; pg 35, para 7-8; pg 38, para 2, 4-6	1-5, 16-19, 33
A	DONOSO et al. Current value of preimplantation genetic aneuploidy screening in IVF. Hum. Reprod. Update January-February 2007 (01-02.2007), Vol. 13, No. 1, pages 15-25	1-5, 16-19, 33
A	STROM et al. Neonatal outcome of preimplantation genetic diagnosis by polar body removal: the first 109 infants. Pediatrics. October 2000 (10.2000), Vol. 106, No. 4, pages 650-653	1-5, 16-19, 33
A	US 2006/0141499 A1 (SHER et al.) 29 June 2006 (29.06.2006)	1-5, 16-19, 33

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 2009 (16.07.2009)	27 JUL 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/52730

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/483, C12Q 1/68 (2009.01)
USPC - 435/6; 702/19
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 33/483, C12Q 1/68 (2009.01)
USPC: 435/6; 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8): G01N 33/483, C12Q 1/68 (2009.01), USPC: 435/6; 702/, 19 - keyword search, as below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Google Scholar, USPTO PubWest (databases: PGPB,USPT,USOC,EPAB,JPAB) - Search Terms: ploidy, ploid, determine, evaluate, assess, measure, ascertain, hypothesis, parent, offspring, related, relative, sibling, maternal, paternal, statistical, probability, combine, sum, add, distribution, allele, phase, gene, gentic, chromosomal, copies, preimplantatio

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2007/0184467 A1 (RABINOWITZ et al.) 9 August 2007 (09.08.07) abstract, para [0068]; [0029]; [0035]; [0036]; [0072]; [0085]; [0075] - [0084]; [0074]; [0119]; [0120]; [0033]; [0140]; [0171]; [0194] - [0197]; [0128]	1-22, 24-26 --- 23
Y	US 2008/0138809 A1 (KAPUR et al.) 12 June 2008 (12.06.2008) para [0217]	23
A	US 2008/0182244 A1 (TAFAS et al.) 31 July 2008 (31.07.2008)	1-26

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2009 (21.09.2009)	28 SEP 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/50824

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; C07H 21/04; G01N 33/50 (2010.01)
USPC - 435/6, 536/23.1, 702/19
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC- C12Q 1/68; C07H 21/04; G01N 33/50 (2010.01);
USPC- 435/6, 536/23.1, 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 702/20;
Patents and NPL

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (US Pat, PgPub, EPO, JPO: classification, keyword), GoogleScholar; search terms: copy number, probability, statistical, compute, algorithm, mathematical, hypothesis, fetal, fetus, foetal, chromosome, polymorphism, snp, ploid, disomal, abort, terminate, end, pregnancy, trimester, paternity, mixed sample, father, mother, paternal, maternal, n

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0243398 A1 (RABINOWITZ et al.) 02 October 2008 (02.10.2008), para [0013]-[0020], [0032], [0040], [0045]-[0050], [0083], [0090]-[0091], [0100], [0107]-[0111], [0226], [0227]-[0233], [0240], [0241], [0268]	1-18
Y	US 2007/0178478 A1 (DHALLAN et al.) 02 August 2007 (02.08.2007), para [0023], [0024], [0027], [0032], [0081], [0233], [0439], [0847]	1-18
A	CHU et al. "Statistical model for whole genome sequencing and its application to minimally invasive diagnosis of fetal genetic disease." Bioinformatics [online], 10 May 2009 (10.05.2009) [Retrieved on 2010-11-03], Vol. 25, Iss. 10, pp. 1244-1250, Retrieved from the Internet: <URL: http://bioinformatics.oxfordjournals.org>	1-18
A	PFAFFL et al. "Relative expression software tool (REST?) for group-wise comparison and statistical analysis of relative expression results in real-time PCR." Nucleic Acids Research [online], May 2009 [Retrieved on 2010-11-03], Vol. 30, Iss. 9, p. e36, 10 pages, Retrieved from the Internet: <URL: http://nar.oxfordjournals.org>	1-18
A	US 2008/0070792 A1 (STOUGHTON et al.) 20 March 2008 (20.03.2008), entire document	1-18
A	PERRY et al. "The Fine-Scale and Complex Architecture of Human Copy-Number Variation." American Journal of Human Genetics [online], 03 March 2008 (03.03.2008) [Retrieved on 2010-11-03], Vol. 82, Iss. 3, pp. 685-695, Retrieved from the Internet: <URL: http://www.sciencedirect.com>	1-18
A	US 2008/0020390 A1 (MITCHELL et al.) 24 January 2008 (24.01.2008), entire document	1-18

☒ Further documents are listed in the continuation of Box C. ☐

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"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 November 2010 (04.11.2010)	15 NOV 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/50824

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2007/0259351 A1 (CHINITZ et al.) 08 November 2007 (08.11.2007), entire document	1-18
A	US 2007/0212689 A1 (BIANCHI et al.) 13 September 2007 (13.09.2007), entire document	1-18
A	US 2007/0207466 A1 (CANTOR et al.) 06 September 2007 (06.09.2007), entire document	1-18
A	SEBAT et al. "Strong Association of De Novo Copy Number Mutations with Autism." Science [online], 20 April 2007 (20.04.2007) [Retrieved on 2008-03-27], Vol. 316, No. 445, pp. 445-449, Retrieved from the Internet: <URL: http://intramural.nimh.nih.gov/>	1-18
A	US 2006/0229823 A1 (LIU et al.) 12 October 2006 (12.10.2006), entire document	1-18
A	US 2006/0121452 A1 (DALLAN et al.) 08 June 2006 (08.06.2006), entire document	1-18
A	US 2006/0057618 A1 (PIPER et al.) 16 March 2006 (16.03.2006), entire document	1-18
A	NANNYA et al. "A Robust Algorithm for Copy Number Detection Using High-Density Oligonucleotide Single Nucleotide Polymorphism Genotyping Arrays." Cancer Research [online], 15 July 2005 (15.07.2005), Vol. 65, No. 14, pp. 6071-6079, Retrieved from the Internet: <URL: http://cancerres.aacrjournals.org>	1-18
A	US 2005/0049793 A1 (PATERLINI-BRECHOT et al.) 03 March 2005 (03.03.2005), entire document	1-18
A	US 2005/0009069 A1 (LIU et al.) 13 January 2005 (13.01.2005), entire document	1-18
A	DURAWALA et al. "A versatile statistical analysis algorithm to detect genome copy number variation." Proceedings of the National Academy of Science [online], 16 November 2004 (16.11.2004) [Retrieved on 2010-11-03], Vol. 101, No. 46, pp. 16292-16297, Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov>	1-18
A	US 2003/0101000 A1 (BADER et al.) 29 May 2003 (29.05.2003), entire document	1-18
A	US 6,180,349 B1 (GINZINGER et al.) 30 January 2001 (30.01.2001), entire document	1-18

NeoGenomics
Exhibit 1003 Page 1062

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/37018

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2011.01)
USPC - 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68 (2011.01)
USPC - 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6; 702/19, 435/91.5
(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB, JPAB); Google Scholar.
Search Terms: ploidy calling, copy number calling, joint distribution, ploidy, heterozygo$, rate, copy number, allel$, polymorphism, SNP, enrich, enrich$, amplif$, probe, forward primer, upstream, reverse primer, downstream, hybrid, captur$, probe, hybridiz$, flank, flanking,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0202525 A1 (QUAKE et al.) 30 August 2007 (30.08.2007) claim 1-2; para [0026]-[0028]. [0056]-[0061], [0126], [0127].	27
Y	WO 2010/017214 A1 (RABINOWITZ et al.) 11 February 2010 (11.02.2010) claim 1, 8-9, 18-19; pg 4, ln 5 to pg 5, ln 15; pg 6, ln 5-25; pg 12, ln 1-10; pg 15, ln 16-21; pg 24, ln 8 to pg 25, ln 32; pg 26, ln 20 to pg 27, ln 25 to pg 28, ln 7, pg 29, ln 13-30; pg 52, ln 1-16; pg 100, ln 27 to pg 101, ln 7.	1-24
Y	GARDINA et al., Ploidy status and copy number aberrations in primary glioblastomas defined by integrated analysis of allelic ratios, signal ratios and loss of heterozygosity using 500K SNP Mapping Arrays. BMC Genomics. 2008 (published online 17 October 2008), Vol 9, No 489, pages 1-16: abstract; pg 3, col 1, para 1 to col 2, para 4.	1-26
Y	US 2005/0255508 A1 (CASEY et al.) 17 November 2005 (17.11.2005) para [0007], [0045]-[0049].	1-24
Y	TURNER et al., Massively parallel exon capture and library-free resequencing across 16 genomes. Nature Methods. May 2009 (published online 6 April 2009), Vol 6, No 5, pages 315-316: fig 1-2.	5, 10 and 25-26
Y	US 2006/0216738 A1 (WADA et al.) 28 September 2006 (28.09.2006) para [0022], [0024], [0041].	6
Y	US 2005/0142577 A1 (JONES et al.) 30 June 2005 (30.06.2005) para [0058], [0059], [0062], [0063], [0081], [0082].	7-9

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 2011 (26.07.2011)	27 SEP 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/37018

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0112590 A1 (LO et al.) 06 May 2010 (06.05.2010) para [0013], [0112].	13

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/61506

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G06F 19/22; G01N 33/48 (2012.01)
USPC - 702/20; 436/63, 94
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)- G06F 19/22; G01N 33/48 (2012.01);
USPC- 702/20; 436/63, 94

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Patents and NPL (classification, keyword; search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (US Pat, PgPub, EPO, JPO), GoogleScholar (PL, NPL), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL); search terms: ploid, fetal, fetus, gestate, state, hypothesis, joint, distribute, probability, likelihood, call, allele, DNA, nucleic, nucleotide, probe, hybrid, ligate, circular, SNP, polymorphism, loci, locus, flank, forward, rever

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0184467 A1 (RABINOWITZ et al.) 09 August 2007 (09.08.2007), para [0029]-[0036], [0039], [0069]-[0079], [0121], [0126], [0127], [0142], [0144], [0148]-[0150], [0171], [0180], [0185], [0206], [0277], [0294], [0323], [0390]	1-43
Y	US 2007/0178478 A1 (DHALLAN et al.) 02 August 2007 (02.08.2007), para [0032], [0033], [0081], [0140], [0216], [1964]	1-42
Y	US 2007/0202525 A1 (QUAKE et al.) 30 August 2007 (30.08.2007), para [0027], [0033], [0055], [0082], [0125], [0126], [0129], [0140]	43
Y	US 2009/0099041 A1 (CHURCH et al.) 16 April 2009 (16.04.2009), para [0008], [0009], [0015], [0019], [0029], [0037]-[0045], [0046], [0051], [0059], [0068], [0071], [0093], [0096], [0101]-[0103], [0109]-[0122], [0125]	5-21, 42
X, P ----- Y, P	WO 2010/017214 A1 (RABINOWITZ et al.) 11 February 2010 (11.02.2010), pg 3, ln 31; pg 4, ln 1-8; pg 4, ln1 2-32; pg 5, ln 15-22; pg 6, ln 11-33; pg 10, ln 26-30; pg 15, ln 5-13; pg 19, ln 1 to pg 20, ln 24; pg 22, ln 1-6; pg 23, ln 24-29; pg 24, ln 32 to pg 25, ln 12; pg 27, ln 1-4; pg 46, ln 5-30; pg 49, ln 15-28; pg 50, ln 1 to pg 51, ln 19; pg 73, ln 2-4; pg 79, ln 16 to pg 80, ln 15; pg 85, ln 15-33; pg 89, ln 12-25; pg 91, ln 3-5; pg 92, ln 3-8; pg 97, ln 19-31; pg 98, ln 4 to pg 99, ln 27; pg 101, ln 1-3	1-4, 22, 27-41 ----- 5-21, 23-26, 42, 43
Y	US 2005/0255508 A1 (CASEY et al.) 17 November 2005 (17.11.2005), para [0007]-[0128]	1-43

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2012 (27.02.2012)	16 MAR 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/66938

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2012.02)
USPC - 435/6.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/6.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/6.11, 6.12, 6.1; 702/20 (keywords below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed: paternity, testing, fetus, fetal, maternal, DNA, blood, mixed, SNP, polymorphism, computer, calculate, statistical, noninvasive, cfDNA, cell-free DNA, prenatal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0071076 A1 (HAHN, et al.) 20 March 2008 (20.03.2008) para [0006], [0036], [0041], [0043], [0045]-[0054], [0056]; Table 2	1-13, 15-25
Y	PHILLIPS, et al. Resolving relationship tests that show ambiguous STR results using autosomal SNPs as supplementary markers. Forensic Sci. Int. Genet. 2008, 2(3):198-204; abstract; pg 199, para 3 - pg 200, para 2; pg 201, para 2; Fig. 1A; Tables 1, 3	1-13, 15-25
Y	HOMER, et al. Resolving individuals contributing trace amounts of DNA to highly complex mixtures using high-density SNP genotyping microarrays. PLoS Genet. 2008, 4(8):e1000167, pages 1-9; abstract; pg 2, para 5 - pg 3, para 3; pg 7, para 7	8-13
Y	US 2010/0216153 A1 (LAPIDUS, et al.) 26 August 2010 (26.08.2010) para [0039]	4-5
A	WAGNER, et al. Non-invasive prenatal paternity testing from maternal blood. Int. J. Legal Med. 2008, 123:75-79	1-13, 15-25

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 2012 (07.06.2012)	20 JUN 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/066339

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07H 21/02, 21/04; C12N 1/08; C12P 19/34; C12Q 1/68; C40B 20/04, 20/08, 40/06, 50/02, 50/06, 50/14, 60/02, 60/14; G01N 33/483, 33/49, 33/50, 33/53 (2013.01)
USPC - 435/6.11, 91.1, 91.2; 436/813, 814; 506/2, 6, 16, 24, 26, 34; 536/2, 23.1, 24.33, 25.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC Class/Subclass(es): B01J 19/0046, 2219/00585, 2219/00707, 2219/00722; C07H 21/00; C12Q 1/686, 1/689, 1/6883

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit.com, Google Patents, Google Scholar, ProQuest

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2012/0122701 A1 (RYAN et al) 17 May 2012 (17.05.2012) entire document	1-27, 33, 40, 41, 43-61, 63-68, 70-102 --- 28-32, 34-39, 42, 62, 69
Y	US 2008/0234142 A1 (LIETZ) 25 September 2008 (25.09.2008) entire document	28-32, 34-39
Y	WO 2001/090419 A2 (STANTON) 29 November 2001 (29.11.2001) entire document	37, 38
Y	US 2005/0227263 A1 (GREEN et al) 13 October 2005 (13.10.2005) entire document	42, 62
Y	US 2006/0210997 A1 (MYERSON et al) 21 September 2006 (21.09.2006) entire document	42, 62
Y	US 6,479,235 B1 (SCHUMM et al) 12 November 2002 (12.11.2002) entire document	69

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2013	05 MAR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DAVID J. DYKEMAN
GREENBERG TRAURIG, LLP
ONE INTERNATIONAL PLACE
BOSTON, MA 02110

RECEIVED
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IP DEPT. BOSTON

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing *(day/month/year)*	28 MAY 2013
Applicant's or agent's file reference 118103-012502/PCT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US2013/028378	International filing date *(day/month/year)* 28 February 2013
Applicant NATERA, INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

   Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450	Authorized officer Blaine R. Copenheaver
Facsimile No. 571-273-3201	Telephone No. PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2010)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 118103-012502/PCT	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US2013/028378	International filing date *(day/month/year)* 28 February 2013	(Earliest) Priority Date *(day/month/year)* 29 February 2012
Applicant NATERA, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

   c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

   a. the figure of the **drawings** to be published with the abstract is Figure No. 27D

   ☒ as suggested by the applicant.

   ☐ as selected by this Authority, because the applicant failed to suggest a figure.

   ☐ as selected by this Authority, because this figure better characterizes the invention.

   b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/028378

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☒ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/028378

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C06F 19/18, 16/20; C12Q 1/68; G01N 33/50 (2013.01)
USPC - 435/6.11, 6.12, 287.2; 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC- C12Q 1/6883 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/0288780 A1 (RABINOWITZ et al) 24 November 2011 (24.11.2011) entire document	1-6, 8-32
Y		7
Y	US 5,972,602 A (HYLAND et al) 26 October 1999 (26.10.1999) entire document	7
A	US 2011/0105353 A1 (LO et al) 05 May 2011 (05.05.2011) entire document	1-32

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 May 2013	28 MAY 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: DAVID J. DYKEMAN
GREENBERG TRAURIG, LLP
ONE INTERNATIONAL PLACE
BOSTON, MA 02110

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	28 MAY 2013
Applicant's or agent's file reference 118103-012502/PCT	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2013/028378	28 February 2013	29 February 2012

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.11

Applicant NATERA, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer:
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	02 May 2013	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2013/026378

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      ☒ on paper

      ☒ in electronic form

   b. (time)

      ☒ in the international application as filed

      ☐ together with the international application in electronic form

      ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No
PCT/US2013/028378

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	7-10, 12, 13	YES
	Claims	1-6, 11, 14-32	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-32	NO
Industrial applicability (IA)	Claims	1-32	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-6, 11, and 14-32 lack novelty under PCT Article 33(2) as being anticipated by Rabinowitz et al. (hereafter Rabinowitz).

Regarding claim 1, Rabinowitz discloses, an ex vivo method for determining a number of copies of a chromosome or chromosome segment of interest in the genome of a fetus (the present disclosure provides ex vivo methods for determining the ploidy status of a chromosome in a gestating fetus, Para. [0038]), the method comprising: making genotypic measurements at a plurality of polymorphic loci on the chromosome or chromosome segment from a sample comprising cellular genetic material from the fetus and/or the mother of the fetus (from genotypic data measured from a mixed sample of DNA ,i.e., DNA from the mother of the fetus, and DNA from the fetus... the mixed sample of DNA is preferentially enriched at a plurality of polymorphic loci, Para. [0038]); obtaining genotypic measurements at the plurality of polymorphic loci from genetic material from the mother (from genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus... the mixed sample of DNA is preferentially enriched at a plurality of polymorphic loci, Para. [0038]; the fraction of fetal DNA, or the percentage of fetal DNA in the mixture can be measured...the fraction can be calculated using only the genotyping measurements made on the maternal plasma sample itself, which is a mixture of fetal and maternal DNA, Para. [0322]); determining, on a computer, based on the genotypic measurements, whether the sample comprises (i) DNA from the fetus with essentially no DNA from the mother, (ii) maternal DNA with essentially no DNA from the fetus, or (iii) DNA from both the mother and the fetus (the fraction of fetal DNA, or the percentage of fetal DNA in the mixture can be measured...the fraction can be calculated using only the genotyping measurements made on the maternal plasma sample itself, which is a mixture of fetal and maternal DNA, Para. [0322]; any of the embodiments disclosed herein may be implemented in... computer hardware, firmware, software, or in combinations thereof, Para. [0132]) and; determining, on a computer based on the genotypic measurements (analyzing the genotypic data, on a computer, and ex vivo, Para. [0047]), the number of copies of a chromosome or chromosome segment in the genome of the fetus when the sample comprises DNA from the fetus (determining a fetal aneuploidy by determining the number of copies of maternal and fetal target chromosomes, Para. [0018]; non-invasive prenatal diagnosis involves a number of steps. Some of the steps may include: (1) obtaining the genetic material from the fetus; (2) enriching the genetic material of the fetus, ex vivo; (3) amplifying the genetic material, ex vivo; (4) preferentially enriching specific loci in the genetic material, ex vivo; (5) genotyping the genetic material, ex vivo; and (6) analyzing the genotypic data, on a computer, and ex vivo, Para. [0047]).

Regarding claim 2, Rabinowitz discloses the method of claim 1. Rabinowitz further discloses wherein the sample comprises DNA from the fetus, and wherein the determining the number of copies of the chromosome further comprises: creating, on a computer (any of the embodiments disclosed herein may be implemented in... computer hardware, firmware, software, or in combinations thereof, Para. [0132]), a set of one or more hypotheses about the number of copies of the chromosome or chromosome segment in the genome of the fetus (one step involves creating a hypothesis. In some embodiments, the hypothesis is a copy number hypothesis, Para. [0136]; copy number hypothesis... refers to a hypothesis concerning the number of copies of a particular chromosome in an individual, Para. [0108]); determining, on a computer (any of the embodiments disclosed herein may be implemented in... computer hardware, firmware, software, or in combinations thereof, Para. [0132]), the probability of each of the hypotheses given the genotypic measurements (once the set of hypotheses have been defined, when the algorithms operate on the input genetic data, they may output a determined statistical probability for each of the hypotheses under consideration, Para. [0141]); and using the probabilities associated with each hypothesis to determine the most likely number of copies of the chromosome or chromosome segment in the genome of the fetus (the probabilities of the various hypotheses may be determined by mathematically calculating, for each of the various hypotheses, the value that the probability equals, as stated by one or more of the expert techniques, algorithms, and/or methods described elsewhere in this disclosure, using the relevant genetic data as input, Para. [0141]; the method disclosed herein can be used to determine a fetal aneuploidy by determining the number of copies of maternal and fetal target chromosomes, Para. [0353]).

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2013/028378

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

Regarding claim 3, Rabinowitz discloses an ex vivo method for determining a number of copies of a chromosome or chromosome segment of interest in the genome of a fetus (the present disclosure provides ex vivo methods for determining the ploidy status of a chromosome, Para. [0038]; methods are disclosed for determining a fetal aneuploidy by determining the number of copies of maternal and fetal target chromosomes, Para. [0018]), the method comprising: making genotypic measurements at a plurality of polymorphic loci on the chromosome or chromosome segment from a mixed sample (from genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus... the mixed sample of DNA is preferentially enriched at a plurality of polymorphic loci, Para. [0038]) that comprises (i) cellular genetic material from the fetus and (ii) cellular genetic material from the mother of the fetus (from genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus... the mixed sample of DNA is preferentially enriched at a plurality of polymorphic loci, Para. [0038]); creating, on a computer any of the embodiments disclosed herein may be implemented in... computer hardware, firmware, software, or in combinations thereof, Para. [0132]), a set of one or more hypotheses about the number of copies of the chromosome or chromosome segment in the genome of the fetus (one step involves creating a hypothesis...in some embodiments , the hypothesis is a copy number hypothesis. Para. [0136]; copy number hypothesis... refers to a hypothesis concerning the number of copies of a particular chromosome in an individual, Para. [0108]); determining, on a computer, the probability of each of the hypotheses given the genotypic measurements (once the set of hypotheses have been defined, when the algorithms operate on the input genetic data, they may output a determined statistical probability for each of the hypotheses under consideration, Para. [0141]); and using the probabilities associated with each hypothesis to determine the most likely number of copies of the chromosome or chromosome segment in the genome of the fetus (the probabilities of the various hypotheses may be determined by mathematically calculating, for each of the various hypotheses, the value that the probability equals, as stated by one or more of the expert techniques, algorithms, and/or methods described elsewhere in this disclosure, using the relevant genetic data as input, Para. [0141]).

Regarding claim 4, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the fetal cellular genetic material is from a gestating fetus (determining the ploidy status of a chromosome in a gestating fetus from genotypic data measured from a mixed sample of DNA, Para. [0038]).

Regarding claim 5, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein fetal cellular genetic material is from a products of conception sample (it is possible to phase the parental genotype from unphased genotypic information using grandparental genetic data, or genetic data from other born children, or a sample of a miscarriage, Para. [0419]).

Regarding claim 6, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the mixed sample is a maternal whole blood sample, cells isolated from a maternal blood sample, amniocentesis sample, placental tissue sample, cervical mucus sample, or sample from a fetus (the mixed sample is derived from maternal blood, Para. [0038]).

Regarding claim 11, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the mixed sample is enriched for fetal cells (obtaining a maternal blood sample, and enriching the fetal DNA. The method may involve amplifying and/or genotyping or sequencing the genetic material in the sample, Paras. [0054] and [0362]).

Regarding claim 14, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses obtaining genotypic measurements at the plurality of polymorphic loci from genetic material from the mother, wherein the probability of each hypotheses is determined using (i) the genotypic measurements made on the genetic material from the mother (obtaining genotypic data from one or both parents of the fetus... building on a computer, a joint distribution model for heterozygosity rates of each polymorphic allele on a chromosome for each ploidy hypothesis using genotypic data from the one or both parents, Para. [0009]) and (ii) the genotypic measurements made on the mixed sample (from genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus)... the mixed sample of DNA is preferentially enriched at a plurality of polymorphic loci, Paras. [0009] and [0038]).

Regarding claim 15, Rabinowitz discloses the method of claim 14. Rabinowitz further discloses wherein the genotypic measurements made on the genetic material from the mother facilitate the detection or quantitation of maternal DNA in the mixed sample (the franction of fetal DNA, or the percentage of fetal DNA in the mixture can be measured...the fraction can be calculated using only the genotyping measurements made on the maternal plasma sample itself, which is a mixture of fetal and maternal DNA, Para. [0322]).

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2013/028378

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

Regarding claim 16, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses obtaining genotypic measurements at the plurality of polymorphic loci from genetic material from the mother and from genetic material from the father (genotypic data measured from a mixed sample of DNA, i.e., DNA from the mother of the fetus, and DNA from the fetus and from genotypic data measured from a sample of genetic material from the mother and optionally also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data, Para. [0038]), wherein the probability of each hypotheses is determined using (i) the genotypic measurements made on the genetic material from the mother (genotypic data measured from a mixed sample of DNA (i.e., DNA from the mother of the fetus, and DNA from the fetus) and from genotypic data measured from a sample of genetic material from the mother and optionally also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data, Para. [0038]), (ii) the genotypic measurements made on the genetic material from the father (genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus and from genotypic data measured from a sample of genetic material from the mother and optionally also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data, Para. [0038]), and (iii) the genotypic measurements made on the mixed sample (genotypic data measured from a mixed sample of DNA i.e., DNA from the mother of the fetus, and DNA from the fetus, and from genotypic data measured from a sample of genetic material from the mother and optionally also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data, Paras. [0038] and [0412]).

Regarding claim 17, Rabinowitz discloses the method of claim 16. Rabinowitz further discloses wherein DNA cross-linking inhibits the measuring of the genetic material from the mixed sample (determining the genetic state of a fetus where the fetal DNA is found in maternal blood, but this example is not meant to limit to possible contexts that this method may be applied to. In addition, the method may be applicable in cases where the amount of target DNA is in any proportion with the non-target DNA; for example, the target DNA could make up anywhere between 0.000001 and 99.999999 percent of the DNA present. In addition, the non-target DNA does not necessarily need to be from one individual, or even from a related individual, as long as genetic data from non-target individual(s) is known. In one embodiment of the present disclosure, the method can be used to determine genotypic data of a fetus from maternal blood that contains fetal DNA. In one embodiment, the method can be used in a case where there are multiple fetuses in the uterus of a pregnant woman, or where other contaminating DNA may be present in the sample, Paras. [0052] and [0364]); and wherein genotypic measurements made on the genetic material from the father facilitate the copy number determination (genotypic data measured from a mixed sample of DNA, i.e., DNA from the mother of the fetus, and DNA from the fetus and from genotypic data measured from a sample of genetic material from the mother and optionally also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data. Paras. [0038] and [0412]).

Regarding claim 18, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the plurality of loci comprise single nucleotide polymorphisms (SNPs) (the set of polymorphic alleles are SNPs, Para. [0011]; the targeting may focus entirely on SNPs, Para. [0158]).

Regarding claim 19, Rabinowitz discloses the method of claim 18. Rabinowitz further discloses wherein plurality of loci comprises at least 100 SNPs (it is possible to focus on a small number of SNP's, for example between 1 and 100 SNPs, or a larger number, for example between 100 and 1,000, between 1,000 and 10,000, between 10,000 and 100,000 or more than 100,000 SNPs, Para. [0158]).

Regarding claim 20, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the probability of each of the hypotheses is determined using data about the probability of chromosomes crossing over at different locations in the chromosome or chromosome segment of interest (building a joint distribution model is done by using data about the probability of chromosomes crossing over at different cross-over locations in a chromosome to model dependence between polymorphic alleles on the chromosome. Para. [0013]).

Regarding claim 21, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the method comprises calculating, on a computer, allele counts at the plurality of polymorphic loci from the genotypic measurements (calculating, on a computer, allele ratios at the set of polymorphic alleles from the DNA measurements made on the second sample, Para. [0009]), building, on a computer, a joint distribution model for the expected allele counts at the plurality of polymorphic loci on the chromosome or chromosome segment for each hypothesis (building, on a computer, a joint distribution model for heterozygosity rates of each polymorphic allele on the chromosome for each ploidy hypothesis using genotypic data from the one or both parents of the fetus, Para. [0009]); determining, on a computer, a relative probability of each of the copy number hypotheses using the joint distribution model and the allele counts (determining, on a computer, a relative probability of each of the ploidy hypotheses using the joint distribution model and the allele ratios calculated for the second sample, Para. [0009]).

Regarding claim 22, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein a SNP genotyping array is used to make the genotypic measurements (parental context may refer to a set or subset of target SNPs, Para. [0147]; isolated DNA can then be measured by a number of means, such as by hybridizing to a genotyping array, Para. [0148]).

Regarding claim 23, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein high throughput DNA sequencing is used to make the genotypic measurements (This isolated DNA can then by measured by a number of means... sequencing on a high throughput sequencer, Para. [0148]).

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

Regarding claim 24, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein a confidence for the copy number determination is at least 95% (checking concordance of hit rates and given confidences, for a range of confidence >90%, Para. [0352]).

Regarding claim 25, Rabinowitz discloses the method of claim 3. Rabinowitz further comprising using the genotypic measurements to determine whether or not the fetus has inherited one or more disease linked haplotypes (the knowledge of the maternal genetic data may be used along with the genetic data measured from the free floating DNA found in the blood to determine which haplotypes from the mother and father are present in the fetus. This knowledge may be used to determine the presence or absence of specific disease related genes or other phenotypically correlated genes in the genotype of the fetus, Para. [0365]).

Regarding claim 26, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses comprising performing a clinical action based on the copy number determination (taking a clinical action based on the called ploidy state of the fetus, Para. [0018]).

Regarding claim 27, Rabinowitz discloses the method of claim 26. Rabinowitz further discloses wherein the clinical action is terminating the pregnancy or maintaining the pregnancy (taking a clinical action based on the called ploidy state of the fetus, wherein the clinical action is selected from one of terminating the pregnancy or maintaining the pregnancy, Para. [0016]).

Regarding claim 28, Rabinowitz discloses the method of claim 26. Rabinowitz further discloses wherein the clinical action is selected from the group consisting of performing amniocentesis on the fetus, performing amniocentesis on a subsequent fetus that that inherits genetic material from the mother and/or father, performing chorion villus biopsy on the fetus, performing chorion villus biopsy on a subsequent fetus that that inherits genetic material from the mother and/or father, performing in vitro fertilization, performing preimplantation genetic diagnosis on one or more embryos that inherited genetic material from the mother and/or father, karyotyping the mother, karyotyping the father, and combinations thereof (the method may be used in the context of in vitro fertilization, where it may be desirable to genotype a single cell blastomere biopsied from a cleavage state embryo for the purposes of determining the genetic state of the embyo, Para. [0189]).

Regarding claim 29, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the chromosome of interest is selected from the group consisting of chromosome 13, chromosome 18, chromosome 21, the X chromosome, the Y chromosome, and combinations thereof (it is possible to use this method to create a DNA sample that is enriched in SNPs located on one or a small number of chromosomes that are correlated with live trisomic births, for example chromosomes 13, 18, 21, X and Y, or some combination thereof, Para. [0157]).

Regarding claim 30, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the method comprises screening for a chromosomal abnormality selected from the group consisting of monosomy, uniparental disomy, trisomy, other aneuploidies, mosaicism, unbalanced translocations, insertions, deletions, and combinations thereof (it may also be used to infer the more complete genotypic information of the fetus, specifically, allele calls, the presence of insertions, deletions, transpositions, and other genetic modifications that may correlate with diseases, conditions, or other phenotypes, Para. [0365]).

Regarding claim 31, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein the method comprises determining whether the individual has Down syndrome, Edwards syndrome, Patau syndrome, Klinefelters syndrome, 47,XXX, 47,XYY, Turner syndrome, triploidy, DiGeorge syndrome, Cri du Chat syndrome, Angelman syndrome, Praeder-Willi syndrome, Wolf-Hirschhorn syndrome, SmithMagenis syndrome, Williams-Beuren syndrome, Phelan-McDermid syndrome, Sotos Syndrome, Cystic Fibrosis, Muscular Dystrophy, Spinal Muscular Atrophy, Fragile X, Tay- Sachs disease, Gaucher disease, Torsion Dystonia, Niemann-Pick disease, Mucolipidosis, Fanconi Anemia, Canavan disease, Sickle Cell Anemia, or Bloom Syndrome (the congenital disorder is a malformation, neural tube defect, chromosome abnormality, Down syndrome, Para. [0393]).

Regarding claim 32, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses a report displaying a copy number determination for a chromosome or chromosome segment of interest in the genome of a fetus generated using the method of claim 3 (the knowledge of the determined ploidy state may be used to make a clinical decision. This knowledge, typically stored as a physical arrangement of matter in a memory device, may then be transformed into a report, Para. [0429];determining a fetal aneuploidy by determining the number of copies of maternal and fetal target chromosomes, Para. [0018]).

Claim 8-10, 12, and 13 lack an inventive step under PCT Article 33(3) as being obvious over Rabinowitz et al (hereafter Rabinowitz)

Regarding claim 8, Rabinowitz discloses the method of claim 3. Rabinowitz further discloses wherein at least 10% of the DNA in the mixed sample are maternal DNA (the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA... the fetal DNA may be mixed with maternal DNA in ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%, Para. [0434]), but fails to explicitly disclose wherein the sample is cells.
However, it would have been obvious to one of ordinary skill in the art at the time the invention was made to utilize a percentage of cells in the sample instead of DNA, since selection of a known material on the basis of its suitability for an intended use involves only routine skill in the art. The motivation for doing so would be to determine the genotype of an individual from a mixed sample of cells (Rabinowitz, There are a number of published methods in the prior art that demonstrate how one may determine the ploidy state of a fetus from a mixed sample of maternal and fetal DNA...these methods target thousands of locations along each chromosome, Para. [0285]; In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood, Para. [0431]).

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2013/028378

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

Regarding claim 9, Rabinowitz discloses the method of claim 8. Rabinowitz further discloses wherein at least 50% of the DNA in the mixed sample are maternal DNA (the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA... the fetal DNA may be mixed with maternal DNA in ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%, Para. [0434]), but fails to explicitly disclose wherein the sample is cells.
However, it would have been obvious to one of ordinary skill in the art at the time the invention was made to utilize a percentage of cells in the sample instead of DNA, since selection of a known material on the basis of its suitability for an intended use involves only routine skill in the art. The motivation for doing so would be to determine the genotype of an individual from a mixed sample of cells (Rabinowitz, There are a number of published methods in the prior art that demonstrate how one may determine the ploidy state of a fetus from a mixed sample of maternal and fetal DNA...these methods target thousands of locations along each chromosome, Para. [0285]; In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood, Para. [0431]).

Regarding claim 10, Rabinowitz discloses the method of claim 9. Rabinowitz further discloses wherein at least 90% of the DNA in the mixed sample are maternal DNA (the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA... the fetal DNA may be mixed with maternal DNA in ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%, Para. [0434]), but fails to explicitly disclose wherein the sample is cells.
However, it would have been obvious to one of ordinary skill in the art at the time the invention was made to utilize a percentage of cells in the sample instead of DNA, since selection of a known material on the basis of its suitability for an intended use involves only routine skill in the art. The motivation for doing so would be to determine the genotype of an individual from a mixed sample of cells (Rabinowitz, There are a number of published methods in the prior art that demonstrate how one may determine the ploidy state of a fetus from a mixed sample of maternal and fetal DNA...these methods target thousands of locations along each chromosome, Para. [0285];In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood, Para. [0431]).

Regarding claim 12, Rabinowitz discloses the method of claim 11. Rabinowitz further discloses wherein at least 1% of the DNA in the mixed sample are fetal DNA (the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA... the fetal DNA may be mixed with maternal DNA in ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%, Para. [0434]), but fails to explicitly disclose wherein the sample is cells.
However, it would have been obvious to one of ordinary skill in the art at the time the invention was made to utilize a percentage of cells in the sample instead of DNA, since selection of a known material on the basis of its suitability for an intended use involves only routine skill in the art. The motivation for doing so would be to determine the genotype of an individual from a mixed sample of cells (Rabinowitz, There are a number of published methods in the prior art that demonstrate how one may determine the ploidy state of a fetus from a mixed sample of maternal and fetal DNA...these methods target thousands of locations along each chromosome, Para. [0285]; In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood, Para. [0431]).

Regarding claim 13, Rabinowitz discloses the method of claim 12. Rabinowitz further discloses wherein at least 4% of the DNA in the mixed sample are fetal DNA (the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA... the fetal DNA may be mixed with maternal DNA in ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%, Para. [0434]), but fails to explicitly disclose wherein the sample is cells.
However, it would have been obvious to one of ordinary skill in the art at the time the invention was made to utilize a percentage of cells in the sample instead of DNA, since selection of a known material on the basis of its suitability for an intended use involves only routine skill in the art. The motivation for doing so would be to determine the genotype of an individual from a mixed sample of cells (Rabinowitz, There are a number of published methods in the prior art that demonstrate how one may determine the ploidy state of a fetus from a mixed sample of maternal and fetal DNA...these methods target thousands of locations along each chromosome, Para. [0285]; In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood, Para. [0431]).

Claim 7 lacks an inventive step under PCT Article 33(3) as being obvious over Rabinowitz et al (hereafter Rabinowitz) in view of Hyland et al. (hereafter Hyland).

Regarding claim 7, Rabinowitz discloses the method of claim 6. Rabinowitz fails to explicitly disclose wherein the placental tissue sample is from chorionic villus, decidua, or placental membrane.
Hyland is in the field of the quantification of copy numbers (abstract) and teaches wherein the placental tissue sample is from chorionic villus, deciduas, or placental membrane (PCR typing of a fetus would be even be possible using any fetal cell containing DNA such as chorionic villi, Col. 7 Lns. 40-41).
It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the method of Rabinowitz to include wherein the placental tissue sample is from chorionic villus as disclosed in Hyland because such a sample can be advantageously amplified in the absence of red blood cells or large quantities of tissue, leading to safer testing (Hyland Col. 7 Lns. 35-39).

Claims 1-32 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference W19	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US14/51926	International filing date *(day/month/year)* 20 August 2014 (20.08.2014)	(Earliest) Priority Date *(day/month/year)* 20 August 2013 (20.08.2013)
Applicant NATERA, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☒ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the drawings to be published with the abstract is Figure No. _____

☐ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☒ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/51926

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/51926

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2014.01)
CPC - C12Q 1/6883, 1/6827, 1/6816
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/68 (2014.01)
CPC: C12Q 1/6883, 1/6827, 1/6816; USPC: 435/6.1, 4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Dialog ProQuest; Entrez Pubmed; 'blood samples,' 'fetal DNA fraction,' percentiles, 'chromosomal abnormalities,' 'ploidy,' 'placental detachment,' amniocentesis, subpopulations

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2013/0196862 A1 (RABINOWITZ, M et al.) August 1, 2013; abstract; paragraphs [0008], [0011], [0029], [0034], [0037], [0105], [0106], [0218], [0232], [0384], [0510]	1, 2 ----- 3, 4
Y	US 2012/0270212 A1 (RABINOWITZ, M et al.) October 25, 2012; paragraphs [0062], [0088]-[0090], [0200], [0508]	3, 4
Y	US 2013/0190653 A1 (ALVAREZ-RAMOS, AG) July 25, 2013; abstract; paragraphs [0033], [0036]	4
A	US 2012/0264121 A1 (RAVA, RP et al.) October 18, 2012; abstract	1-4

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2014 (17.11.2014)	09 DEC 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Scott Bortner
201 Industrial Road
Suite 410
San Carlos, California 94070
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	09 DEC 2014
Applicant's or agent's file reference: W19	FOR FURTHER ACTION: See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US14/51926	20 August 2014 (20.08.2014)	20 August 2013 (20.08.2013)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68 (2014.01)
CPC - C12Q 1/6883, 1/6827, 1/6816

Applicant: NATERA, INC.

1. This opinion contains indications relating to the following items:

- [x] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [x] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [x] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1 *bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to th IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Fom PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer:
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	17 November 2014 (17.11.2014)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US14/51926

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
   - ☒ the international application in the language in which it was filed.
   - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
   a. (means)
      - ☐ on paper
      - ☐ in electronic form
   b. (time)
      - ☐ in the international application as filed
      - ☐ together with the international application in electronic form
      - ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US14/51926

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-14

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 5-14 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 5-14 are dependent claims which are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 5-14

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit

- ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US14/51926

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	2-4	YES
	Claims	1	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-4	NO
Industrial applicability (IA)	Claims	1-4	YES
	Claims	NONE	NO

2. Citations and explanations:

Claim 1 lacks novelty under PCT Article 33(2) as being anticipated by US 2013/0196862 A1 to Rabinowitz, et al. (hereinafter 'Rabinowitz'862').

Regarding claim 1, Rabinowitz'862 discloses a method of evaluating a fetus (determining ploidy status of a fetus; abstract; paragraph [0008]) for genetic abnormalities (screening for chromosomal abnormalities; paragraph [0029]), comprising, obtaining a blood sample from a pregnant human subject (blood sample from pregnant mother; paragraphs [0011], [0114]), measuring (paragraph [0106]) the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) in the blood sample (paragraphs [0011], [0114]), determining if the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA in the blood sample (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) is within the lowest 1 percentile of a reference population (fetal fraction is 1% or less of compared to fraction of fetal DNA in individuals with disomic reference chromosomes; paragraph [0218]), and generating a report (generating a ploidy status report; paragraphs [0232], [0510]) indicating that an invasive genetic analysis procedure should be performed (report with results for determining need for amniocentesis; paragraph [0034]).

Claim 2 lacks an inventive step under PCT Article 33(3) as being obvious over Rabinowitz'862.

Regarding claim 2, Rabinowitz'862 discloses a method of evaluating a fetus (determining ploidy status of a fetus; abstract; paragraph [0008]) for genetic abnormalities (screening for chromosomal abnormalities; paragraph [0029]), comprising, obtaining a blood sample from a pregnant human subject (blood sample from pregnant mother; paragraphs [0011], [0114]), measuring (paragraph [0106]) the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) in the blood sample (paragraphs [0011], [0114]), determining if the ratio (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA in the blood sample to maternal cell free DNA in the blood sample (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) is in lowest 1 percentile of a reference population (fetal fraction is 1% or less of compared to fraction of fetal DNA in individuals with disomic reference chromosomes; paragraph [0218]), and performing an invasive genetic test (performing amniocentesis; paragraph [0034]). Rabinowitz'862 does not disclose, in a single, concise embodiment, wherein the invasive genetic test is performed if the fetal fraction is in lowest 1 percentile of a reference population. However, since Rabinowitz'862 discloses determining a fetal fraction in lowest 1 percentile of a reference and performing confirmative, invasive studies, such as amniocentesis, both as disclosed above, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have implemented the use of the separate disclosures of Rabinowitz'862, for including the invasive test step if warranted by results indicative of a fetal DNA fraction, which is in lowest 1 percentile of a reference population, through routine experimentation and testing.

-***-Continued on Next Supplemental Page-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US14/51926

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V;
-Continued from Citations and Explanations:

Claim 3 lacks an inventive step under PCT Article 33(3) as being obvious over Rabinowitz'682 in view of US 2012/0270212 A1 to Rabinowitz et al. (hereinafter 'Rabinowitz'212').

Regarding claim 3, Rabinowitz'862 discloses a method of evaluating a fetus (determining ploidy status of a fetus; abstract; paragraph [0008]) for genetic abnormalities (screening for chromosomal abnormalities; paragraph [0029]), comprising, obtaining a blood sample from a pregnant human subject (blood sample from pregnant mother; paragraphs [0011], [0114]), measuring (paragraph [0106]) the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) in the blood sample (paragraphs [0011], [0114]), determining if the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA in the blood sample (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) is within the lowest 1 percentile of a reference population (fetal fraction is 1% or less of compared to fraction of fetal DNA in individuals with disomic reference chromosomes; paragraph [0218]), and performing an invasive genetic test (performing amniocentesis; paragraph [0034]) if the fetal fraction is in lowest 1 percentile of a reference population (report with results for determining need for amniocentesis; paragraph [0034]; if fetal DNA is low in a mixed sample; paragraph [0105]). Rabinowitz'682 does not disclose the steps of: obtaining a plurality blood samples from a test population of pregnant human subjects, measuring the fetal fraction of the cell free fetal DNA in the blood samples, determining a population subset consisting of members of the test population that have fetal fraction that is in the lowest 1 percentile of the test population, and performing an invasive genetic test on at least one member of the population subset. Rabinowitz'212 discloses obtaining a plurality blood samples from a test population of pregnant human subjects (blood samples derived from four euploid and two trisomy-indicated pregnancies; paragraph [0508]), and measuring the fetal fraction of the cell free fetal DNA in the blood samples (estimating fetal DNA fractions; paragraph [0062]). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Rabinowitz'682, for implementing the step of determining a population subset consisting of members of the test population that have fetal fraction that is in the lowest 1 percentile of the test population, and performing an invasive genetic test on at least one member of the population subset, since the data on the cell-free fetal fraction of DNA obtained from analysis of a plurality of blood samples from a test population of pregnant human subjects, as previously disclosed by Rabinowitz'212, would have allowed individuals exhibiting fetal fractions in the lowest 1 percentile to be selected for performing an invasive genetic test, as previously disclose by Rabinowitz'682 (paragraph [0034]).

Claim 4 lacks an inventive step under PCT Article 33(3) as being obvious over Rabinowitz'682 and further in view of Rabinowitz'212 and US 2013/0190653 A1 (ALVAREZ-RAMOS).

Regarding claim 4, Rabinowitz'682 discloses a method of evaluating a fetus (determining ploidy status of a fetus; abstract; paragraph [0008]) for genetic abnormalities (screening for chromosomal abnormalities; paragraph [0029]), comprising, obtaining a blood sample from a pregnant human subject (blood sample from pregnant mother; paragraphs [0011], [0114]), measuring (paragraph [0106]) the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) in the blood sample (paragraphs [0011], [0114]), and determining if the fetal fraction (fraction or ratio of fetal DNA to maternal DNA; paragraphs [0037], [0218], [0384]) of cell free fetal DNA in the blood sample (free-floating DNA in mixed sample of maternal and fetal DNA; paragraph [0106]) is within the lowest 1 percentile of a reference population (fetal fraction is 1% or less of compared to fraction of fetal DNA in individuals with disomic reference chromosomes; paragraph [0218]). Rabinowitz'682 does not disclose obtaining a plurality blood samples from a test population of pregnant human subjects, measuring the fetal fraction of the cell free fetal DNA in the blood sample to maternal cell free DNA in the blood samples, determining a population subset consisting of members of the test population that have fetal fraction that is in the lowest 1 percentile of the test population, and performing a test for a detached placenta on at least one member of the population subset. Rabinowitz'212 discloses obtaining a plurality blood samples from a test population of pregnant human subjects (blood samples derived from four euploid and two trisomy-indicated pregnancies; paragraph [0508]), and measuring the fetal fraction of the cell free fetal DNA in the blood samples (estimating fetal DNA fractions; paragraph [0062]). Alvarez-Ramos discloses blood sample collections appropriate for pregnancies susceptible to placental detachment (blood sampling to prevent placental detachment; abstract; paragraphs [0033], [0036]). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Rabinowitz'682, for implementing the steps of determining a population subset consisting of members of the test population that have fetal fraction that is in the lowest 1 percentile of the test population, and performing a test for a detached placenta on at least one member of the population subset, since the data on the cell-free fetal fraction of DNA obtained from analysis of a plurality of blood samples from a test population of pregnant human subjects, as disclosed by Rabinowitz'212, would have allowed individuals exhibiting fetal fractions in the lowest 1 percentile to be selected for testing for a detached placenta, as disclosed by Alvarez-Ramos, on at least one member of the population subset, as determined by the analysis for low fetal DNA fractions, as previously disclosed by Rabinowitz'682.

Claims 1-4 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

Electronic Acknowledgement Receipt

EFS ID:	40483794
Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
Filer Authorized By:	Anton John Bokal
Attorney Docket Number:	N.012.US.32
Receipt Date:	10-SEP-2020
Filing Date:	21-JUL-2020
Time Stamp:	17:58:49
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

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<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	40483791
Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
Filer Authorized By:	Anton John Bokal
Attorney Docket Number:	N.012.US.32
Receipt Date:	10-SEP-2020
Filing Date:	21-JUL-2020
Time Stamp:	17:58:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

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31	Non Patent Literature	Merriam-WebsterUniversal.pdf	212114 21bc7f3b250c3e8f7c5b08b8b65ae4a69a196906	no	3
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49	Non Patent Literature	NATERA_First_Amended_Answer_2018_28pgs_NPL.pdf	477154 44f0bd9bcb08dd6a3b143aeeb52b62f2b90b5568	no	28
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51	Non Patent Literature	NATERA_Motion_to_Dismiss_16MAY2019_NPL.pdf	91871 c4b7929f7ecec3ccd1b521b0d2a2f6744b68bb33	no	2
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52	Non Patent Literature	NATERA_Opening_Brief_in_Support_of_Motion_to_Dismiss_16MAY2019_NPL.pdf	309220 a6c6ed4e788e77662665a144d6d7ea88caf889ee	no	26
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54	Non Patent Literature	NATERA_Supplemental_Objections_2019_29pgs_NPL.pdf	316458 b6f5924262c76de82e26fdd0f8abda92c82fc693	no	29
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55	Non Patent Literature	Nateras_Rule_3_3_Invalidity_Contentions_and_Rule_3_4_Production_dated_20AUG2018.pdf	340104 8e615845184d726ebeb8e74583b122418021e312	no	17
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57	Non Patent Literature	NGUYEN_DUMONT_2013_69_NPL.pdf	865466 32ea1676a62102c8294785b76f165488c18bc361	no	5
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59	Non Patent Literature	NICOLAIDES_Prenatal_Detection.pdf	114356 817ecbafe4c8acccc8661d4219043b1f464df9540	no	6
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		Total Files Size (in bytes):	87377541		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	40483783
Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
Filer Authorized By:	Anton John Bokal
Attorney Docket Number:	N.012.US.32
Receipt Date:	10-SEP-2020
Filing Date:	21-JUL-2020
Time Stamp:	17:56:21
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

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1	Foreign Reference	CN1650032A_WO03074723A2_1of2.pdf	17005481 0c849da6e851b677ac2a1ff1cb6067d16266e524	no	329

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	40483790
Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
Filer Authorized By:	Anton John Bokal
Attorney Docket Number:	N.012.US.32
Receipt Date:	10-SEP-2020
Filing Date:	21-JUL-2020
Time Stamp:	17:57:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

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7	Non Patent Literature	CARNEVALE_1998.pdf	1347513 579dd3606124dc46e3e26909408b5ed31fee1ad7	no	6
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8	Non Patent Literature	CARVALHO_2007_485_NPL.pdf	734555 3b9346f134b2b9fd396a5d91a759e60ee4df9fb8	no	15
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9	Non Patent Literature	CASBON_2011.pdf	774155 54ca82a4ab72a7f9cb1f33a582692b347251635a	no	8
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New Applications Under 35 U.S.C. 111

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New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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	Art Unit	To be assigned
	Examiner Name	To be assigned
	Attorney Docket Number	N.012.US.32

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	Filing Date	Jul 21, 2020
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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making

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reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Tianran Yan/	Date (YYYY-MM-DD)	2020-09-10
Name/Print	Tianran Yan	Registration Number	67,162

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
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Filing Date:	21-JUL-2020
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Application Type:	Utility under 35 USC 111(a)

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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38	Non Patent Literature	PIRKER_2004_p26_NPL.pdf	1112000 4670128f86e38b22ecf145691bd62ecdba050ab9	no	9
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/934,407	07/21/2020	Joshua BABIARZ	N.012.US.32	2396

110711 7590 07/30/2020

Natera, Inc.
201 Industrial Road
Suite 410
San Carlos, CA 94070

EXAMINER	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
07/30/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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IP@natera.com

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 16/934,407	**Applicant(s)** BABIARZ et al.	
	Examiner CHERYL P GIBSON BAYLOR	**Art Unit** OPET	**AIA (FITF) Status** Yes

1. THE REQUEST FILED 21 July 2020 IS **GRANTED** .

The above-identified application has met the requirements for prioritized examination

A. ☑ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination**. The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;

B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims** , or a multiple dependent claim;

C. filing a **request for continued examination** ;

D. filing a notice of appeal;

E. filing a request for suspension of action;

F. mailing of a notice of allowance;

G. mailing of a final Office action;

H. completion of examination as defined in 37 CFR 41.102; or

I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to CHERYL GIBSON BAYLOR at (571)272-3213. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/CHERYL GIBSON BAYLOR/ Paralegal Specialist, OPET	

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/934,407

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	15 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						2000
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	3720

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/934,407	07/21/2020	1674	3720	N.012.US.32	15	1

CONFIRMATION NO. 2396

110711
Natera, Inc.
201 Industrial Road
Suite 410
San Carlos, CA 94070

FILING RECEIPT

OC000000118773248

Date Mailed: 07/29/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

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Applicant(s)

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Assignment For Published Patent Application

Natera, Inc., San Carlos, CA

Power of Attorney: The patent practitioners associated with Customer Number 110711

Domestic Priority data as claimed by applicant

This application is a CON of 16/743,724 01/15/2020 PAT 10731220
which is a CON of 16/399,268 04/30/2019 PAT 10538814
which is a CON of 16/140,298 09/24/2018
which is a CON of 14/918,544 10/20/2015 PAT 10316362
which is a CIP of 14/877,925 10/07/2015 ABN

and is a CIP of 14/692,703 04/21/2015 PAT 10179937
and is a CIP of 14/538,982 11/24/2014 PAT 9677118
and claims benefit of 62/148,173 04/15/2015
and claims benefit of 62/147,377 04/14/2015
and claims benefit of 62/146,188 04/10/2015
and claims benefit of 62/066,514 10/21/2014
and said 14/877,925 10/07/2015
is a CIP of 14/225,356 03/25/2014 ABN
and is a CIP of 13/780,022 02/28/2013 ABN
and is a CON of 13/683,604 11/21/2012 ABN
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and claims benefit of 61/987,407 05/01/2014
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claims benefit of 61/516,996 04/12/2011

and claims benefit of 61/448,547 03/02/2011
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and claims benefit of 61/462,972 02/09/2011
and claims benefit of 61/398,159 06/21/2010
and claims benefit of 61/395,850 05/18/2010

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 07/27/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/934,407**

Projected Publication Date: 11/05/2020

Non-Publication Request: No

Early Publication Request: No
Title

METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

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For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 16934407 Document Date: 07/21/2020

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 16934407 Document Date: 07/21/2020

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	To be assigned
Filing Date	July 21, 2020
First Named Inventor	Joshua BABIARZ
Title	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
Art Unit	To be assigned
Examiner Name	To be assigned
Attorney Docket Number	N.012.US.32

SIGNATURE of Applicant or Patent Practitioner

Signature	/Anton Bokal/	Date (Optional)	
Name	Anton Bokal	Registration Number	51,243
Title (if Applicant is a juristic entity)	Chief IP Counsel		
Applicant Name (if Applicant is a juristic entity)	Natera, Inc.		

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ *Total of 2 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 110711

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Natera, Inc.

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	/Anton Bokal/	Date (Optional)	
Name	Anton Bokal		
Title	Chief IP Counsel		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 2 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Filer:	Anton John Bokal/Angela McGowan
Attorney Docket Number:	N.012.US.32

Filed as Large Entity

Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
UTILITY APPL SIZE FEE PER 50 SHEETS >100	1081	5	400	2000
Claims:				
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**7860**

Electronic Acknowledgement Receipt

EFS ID:	40061289
Application Number:	16934407
International Application Number:	
Confirmation Number:	2396
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI
First Named Inventor/Applicant Name:	Joshua BABIARZ
Customer Number:	110711
Filer:	Anton John Bokal/Angela McGowan
Filer Authorized By:	Anton John Bokal
Attorney Docket Number:	N.012.US.32
Receipt Date:	21-JUL-2020
Filing Date:	
Time Stamp:	14:22:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$7860
RAM confirmation Number	E20207KE22495536
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	TrackOne Request	01_N012US32_Track_1_Request_SIGNED.pdf	149843 c5e7175b7809e7953909a2277079d81653fbe25a	no	2
Warnings:					
Information:					
2	Application Data Sheet	02_N012US32_ADS_SIGNED.pdf	1258398 69613ead14d1a9a57e0f7b0521a0219790968654	no	16
Warnings:					
Information:					
3		03_N012US32_Specification.pdf	1959080 151f52dbe755f3449b5b61236ed712e58b4d3002	yes	345

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	341
Claims	342	344
Abstract	345	345

Warnings:					
Information:					
4	Drawings-other than black and white line drawings	04_N012US32_Drawings.pdf	2544338 786ef1010101ddd62f5f6ba487b69de555913a08	no	58
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5	Oath or Declaration filed	05_N012US32_Declaration.pdf	3333942 8bdb1a1c9ec027252b75e85be4d461060aa25f9f	no	20

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6	Power of Attorney	06_N012US32_POA.pdf	229371 3ba60eb0a51ac27e6ccec8e34188fcbaf02c40ed	no	3
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Information:					
7	Sequence Listing (Text File)	N012US32_SL.txt	8725977	no	-
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8	Fee Worksheet (SB06)	fee-info.pdf	41897 ef9df8ad36c254a3a35a3eb5904760dba1d59642	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	18242846		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	BABIARZ, Joshua	Nonprovisional Application Number (if known):	To be assigned
Title of Invention:	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☑ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Tianran Yan/	Date 7-21-2020
Name (Print/Typed) Tianran Yan	Practitioner Registration Number 67162

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☑ *Total of 1 forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Joshua		BABIARZ	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Castro Valley	CA	US

Mailing Address of Inventor:

Address 1	201 Industrial Road
Address 2	Suite 410

City	State/Province	Postal Code	Country
San Carlos	CA	94070	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Tudor	Pompiliu	CONSTANTIN	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Berkeley	CA	US

Mailing Address of Inventor:

Address 1	201 Industrial Road
Address 2	Suite 410

City	State/Province	Postal Code	Country
San Carlos	CA	94070	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Lane	A.	EUBANK	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

City	San Carlos	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	State/Province	CA
Postal Code	94070	Country	US

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	George		GEMELOS	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Portland	State/Province	OR	Country of Residence	US

Mailing Address of Inventor:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	State/Province	CA
Postal Code	94070	Country	US

Inventor 5 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Matthew	Micah	HILL	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Belmont	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	State/Province	CA
Postal Code	94070	Country	US

Inventor 6 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Huseyin	Eser	KIRKIZLAR	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Los Angeles	State/Province	CA	Country of Residence	US

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Mailing Address of Inventor:

Address 1	201 Industrial Road
Address 2	Suite 410

City	San Carlos	State/Province	CA
Postal Code	94070	Country i	US

Inventor 7 — Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Matthew		RABINOWITZ	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	San Francisco	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	201 Industrial Road
Address 2	Suite 410

City	San Carlos	State/Province	CA
Postal Code	94070	Country i	US

Inventor 8 — Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Onur		SAKARYA	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Redwood City	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	201 Industrial Road
Address 2	Suite 410

City	San Carlos	State/Province	CA
Postal Code	94070	Country i	US

Inventor 9 — Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Styrmir		SIGURJONSSON	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	San Jose	State/Province	CA	Country of Residence i	US

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Mailing Address of Inventor:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	**State/Province**	CA
Postal Code	94070	**Country** i	US

Inventor 10 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Bernhard		ZIMMERMANN	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Manteca	**State/Province**	CA	**Country of Residence** i	US

Mailing Address of Inventor:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	**State/Province**	CA
Postal Code	94070	**Country** i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	110711		
Email Address	IP@Natera.com	Add Email	Remove Email

Application Information:

Title of the Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI		
Attorney Docket Number	N.012.US.32	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	58	**Suggested Figure for Publication (if any)**	

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	110711		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	16743724	2020-01-15

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
16743724	Continuation of	16399268	2019-04-30	10538814	2020-01-21
Prior Application Status	Pending				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
16399268	Continuation of	16140298	2018-09-24		
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
16140298	Continuation of	14918544	2015-10-20	10316362	2019-06-11
Prior Application Status	Abandoned				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14918544	Continuation in part of	14877925	2015-10-07		
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14918544	Continuation in part of	14692703	2015-04-21	10179937	2019-01-15
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14918544	Continuation in part of	14538982	2014-11-24	9677118	2017-06-13
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14918544	Claims benefit of provisional	62148173	2015-04-15		
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14918544	Claims benefit of provisional	62147377	2015-04-14		
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14918544	Claims benefit of provisional	62146188	2015-04-10		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14918544	Claims benefit of provisional	62066514	2014-10-21
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14877925	Continuation in part of	14225356	2014-03-25
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14877925	Continuation in part of	13780022	2013-02-28
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14877925	Continuation of	13683604	2012-11-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	62148173	2015-04-15
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	62147377	2015-04-14
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	62146188	2015-04-10
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	62066514	2014-10-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	61994791	2014-05-16

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	61987407	2014-05-01
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14692703	Claims benefit of provisional	61982245	2014-04-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14538982	Claims benefit of provisional	62066514	2014-10-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14538982	Claims benefit of provisional	61994791	2014-05-16
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14538982	Claims benefit of provisional	61987407	2014-05-01
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14538982	Claims benefit of provisional	61982245	2014-04-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14225356	Continuation of	PCTUS2012058578	2012-10-03
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13780022	Continuation in part of	13683604	2012-11-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13780022	Continuation in part of	PCTUS201258578	2012-10-03

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13780022	Continuation in part of	13335043	2011-12-22	10113196	2018-10-30
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13780022	Continuation in part of	13300235	2011-11-18	10017812	2018-07-10
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13780022	Continuation in part of	13110685	2011-05-18	8825412	2014-09-02
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
13780022	Claims benefit of provisional	61634431	2012-02-29		
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13683604	Continuation in part of	13300235	2011-11-18	10017812	2018-07-10
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13683604	Continuation in part of	13110685	2011-05-18	8825412	2014-09-02
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
13683604	Claims benefit of provisional	61675020	2012-07-24		
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
PCTUS201258578	Continuation in part of	13300235	2011-11-18	10017812	2018-07-10
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
PCTUS201258578	Claims benefit of provisional	61683331	2012-08-15		
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
PCTUS201258578	Claims benefit of provisional	61542508	2011-10-03		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13335043	Continuation in part of	13300235	2011-11-18	10017812	2018-07-10

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13335043	Continuation in part of	13110685	2011-05-18	8825412	2014-09-02

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13335043	Claims benefit of provisional	61426208	2010-12-22

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13300235	Continuation in part of	13110685	2011-05-18	8825412	2014-09-02

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13300235	Claims benefit of provisional	61542508	2011-10-03

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13300235	Claims benefit of provisional	61571248	2011-06-23

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13110685	Claims benefit of provisional	61516996	2011-04-12

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13110685	Claims benefit of provisional	61448547	2011-03-02

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13110685	Claims benefit of provisional	61462972	2011-02-09

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13110685	Claims benefit of provisional	61398159	2010-06-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13110685	Claims benefit of provisional	61395850	2010-05-18
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.			Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)
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Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Natera, Inc.		
Mailing Address Information For Applicant:			
Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	**State/Province**	CA
Country	US	Postal Code	94070
Phone Number	650-249-9091	Fax Number	
Email Address	IP@Natera.com		

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
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Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

Organization Name	Natera, Inc.

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1	201 Industrial Road		
Address 2	Suite 410		
City	San Carlos	**State/Province**	CA
Country i	US	Postal Code	94070
Phone Number	650-249-9091	Fax Number	
Email Address	IP@Natera.com		

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Tianran Yan/			Date (YYYY-MM-DD)	2020-07-21
First Name	Tianran	Last Name	Yan	Registration Number	67162

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	N.012.US.32
	Application Number	
Title of Invention	METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

METHODS FOR SIMULTANEOUS AMPLIFICATION OF TARGET LOCI

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Utility Application Ser. No. 16/743,724, filed Jan. 15, 2020.

[0002] U.S. Utility Application Ser. No. 16/743,724 is a continuation of U.S. Utility Application Ser. No. 16/399,268 (now U.S. Patent 10,538,814), filed Apr. 30, 2019.

[0003] U.S. Utility Application Ser. No. 16/399,268 (now U.S. Patent 10,538,814) is a continuation of U.S. Utility Application Ser. No. 16/140,298, filed Sept. 24, 2018.

[0004] U.S. Utility Application Ser. No. 16/140,298 is a continuation of U.S. Utility Application Ser. No. 14/918,544 (now U.S. Patent 10,316,362), filed October 20, 2015.

[0005] U.S. Utility Application Ser. No. 14/918,544 (now U.S. Patent 10,316,362), filed October 20, 2015, is a continuation-in-part application of U.S. Utility Application Serial No. 14/877,925, filed October 7, 2015, now abandoned; a continuation-in-part application of U.S. Utility Application Serial No. 14/692,703, filed April 21, 2015, now U.S. Patent No. 10,179,937; a continuation-in-part application of U.S. Utility Application Serial No. 14/538,982, now U.S. Patent No. 9,677,118, filed November 24, 2014; and claims the benefit of and priority to U.S. Provisional Application Ser. No. 62/148,173, filed April 15, 2015; U.S. Provisional Application Ser. No. 62/147,377, filed April 14, 2015; U.S. Provisional Application Ser. No. 62/146,188, filed April 10, 2015; and U.S. Provisional Application Ser. No. 62/066,514, filed October 21, 2014.

[0006] U.S. Utility Application Serial No. 14/877,925, filed October 7, 2015, now abandoned, is a continuation-in-part of U.S. Utility Application Serial No. 14/225,356 (now Abandoned), filed March 25, 2014; is a continuation-in-part of U.S. Utility Application Serial No. 13/780,022 (now Abandoned), filed Feb. 28, 2013; and is a continuation of U.S. Utility Application Serial No. 13/683,604 (now Abandoned), filed November 21, 2012.

[0007] U.S. Utility Application Serial No. 14/692,703, filed April 21, 2015, now U.S. Patent No. 10,179,937, claims the benefit of and priority to U.S. Provisional Application Ser. No. 62/148,173, filed April 15, 2015; U.S. Provisional Application Ser. No. 62/147,377, filed April 14, 2015; U.S. Provisional Application Ser. No. 62/146,188, filed April 10, 2015; U.S.

Provisional Application Ser. No. 62/066,514, filed October 21, 2014; U.S. Provisional Application Ser. No. 61/994,791, filed May 16, 2014; U.S. Provisional Application Ser. No. 61/987,407, filed May 1, 2014; and U.S. Provisional Application Ser. No. 61/982,245, filed April 21, 2014.

[0008] U.S. Utility Application Serial No. 14/538,982, now U.S. Patent No. 9,677,118, filed November 24, 2014 claims the benefit of and priority to U.S. Provisional Application Ser. No. 62/066,514, filed October 21, 2014; U.S. Provisional Application Ser. No. 61/994,791, filed May 16, 2014; U.S. Provisional Application Ser. No. 61/987,407, filed May 1, 2014; and U.S. Provisional Application Ser. No. 61/982,245, filed April 21, 2014.

[0009] U.S. Utility Application Serial No. 14/225,356 (now Abandoned), filed March 25, 2014 is a continuation of PCT Application PCT/US2012/58578, filed October 3, 2012.

[0010] U.S. Utility Application Serial No. 13/780,022 (now Abandoned), filed February 28, 2013, is a continuation-in-part of U.S. Utility Application Serial No. 13/683,604 (now Abandoned), filed November 21, 2012; a continuation-in-part of PCT Application No. PCT/US2012/58578, filed Oct. 3, 2012; a continuation-in-part of U.S. Utility Application Serial No. 13/335,043, filed Dec. 22, 2011, now U.S. Patent No. 10,113,196; a continuation-in-part of U.S. Utility Application Serial No. 13/300,235, filed November 18, 2011, now U.S. Patent No. 10,017,812; and an continuation-in-part of U.S. Utility Application Serial No. 13/110,685 (now U.S. Patent No. 8,825,412), filed May 18, 2011, and also claims the benefit of U.S. Provisional Application Serial No. 61/634,431, filed February 29, 2012.

[0011] U.S. Utility Application Serial No. 13/683,604 (now Abandoned), filed November 21, 2012, is a continuation-in-part of U.S. Utility Application Serial No. 13/300,235 (now U.S. Patent No. 10,017,812), filed Nov. 18, 2011; is a continuation-in-part of U.S. Utility Application Serial No. 13/110,685 (now U.S. Patent No. 8,825,412), filed May 18, 2011; and claims the benefit of and priority to U.S. Provisional Application Ser. No. 61/675,020, filed July 24, 2012.

[0012] PCT Application No. PCT/US2012/058578, filed October 3, 2012, is a continuation-in-part of U.S. Utility Application Serial No. 13/300,235 (now U.S. Patent No. 10,017,812), filed November 18, 2011; and claims the benefit of and priority to U.S. Provisional Application Ser. No. 61/683,331, filed August 15, 2012; and U.S. Provisional Application Ser. No. 61/542,508, filed October 3, 2011.

[0013] U.S. Utility Application Serial No. 13/335,043, filed December 22, 2011, is a continuation-in-part of U.S. Utility Application Serial No. 13/300,325 (now Patent No.

10,017,812), filed November 18, 2011; a continuation-in-part of U.S. Utility Application Serial No. 13/110,685 (now U.S. Patent No. 8,825,412), filed May 18, 2011; and claims the benefit of U.S. Provisional Application Serial No. 61/426,208, filed December 22, 2010.

[0014] U.S. Utility Application Serial No. 13/300,235 (now U.S. Patent No. 10,017,812), filed November 18, 2011 is a continuation-in-part of U.S. Utility Application Serial No. 13/110,685 (now U.S. Patent No. 8,825,412), filed May 18, 2011; and claims the benefit of U.S. Provisional Application Serial No. 61/542,508, filed October 3, 2011; and U.S. Provisional Application Serial No. 61/571,248, filed June 23, 2011.

[0015] U.S. Utility Application Serial No. 13/110,685 (now U.S. Patent No. 8,825,412), filed May 18, 2011, claims the benefit of U.S. Provisional Application Serial No. 61/516,996, filed April 12, 2011; U.S. Provisional Application Serial No. 61/448,547, filed March 2, 2011; U.S. Provisional Application Serial No. 61/462,972, filed February 9, 2011; U.S. Provisional Application Serial No. 61/398,159, filed June 21, 2010; and U.S. Provisional Application Serial No. 61/395,850, filed May 18, 2010.

[0016] Each of these applications cited above is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0017] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 8, 2020, is named N012US32_SL.txt and is 8,726 kilo bytes in size.

FIELD OF THE INVENTION

[0018] The present invention generally relates to methods and compositions for simultaneously amplifying multiple nucleic acid regions of interest in one reaction volume.

BACKGROUND OF THE INVENTION

[0019] To increase assay throughput and allow more efficient use of nucleic acid samples, simultaneous amplification of many target nucleic acids in a sample of interest can be carried out by combining many oligonucleotide primers with the sample and then subjecting the

sample to polymerase chain reaction (PCR) conditions in a process known in the art as multiplex PCR. Use of multiplex PCR can significantly simplify experimental procedures and shorten the time required for nucleic acid analysis and detection. However, when multiple pairs are added to the same PCR reaction, non-target amplification products may be generated, such as amplified primer dimers. The risk of generating such products increases as the number of primers increases. These non-target amplicons significantly limit the use of the amplified products for further analysis and/or assays. Thus, improved methods are needed to reduce the formation of non-target amplicons during multiplex PCR.

[0020] Improved multiplex PCR methods would be useful for a variety of application, such as Non-Invasive Prenatal Genetic Diagnosis (NPD). In particular, current methods of prenatal diagnosis can alert physicians and parents to abnormalities in growing fetuses. Without prenatal diagnosis, one in 50 babies is born with serious physical or mental handicap, and as many as one in 30 will have some form of congenital malformation. Unfortunately, standard methods have either poor accuracy, or involve an invasive procedure that carries a risk of miscarriage. Methods based on maternal blood hormone levels or ultrasound measurements are non-invasive, however, they also have low accuracies. Methods such as amniocentesis, chorion villus biopsy and fetal blood sampling have high accuracy, but are invasive and carry significant risks. Amniocentesis was performed in approximately 3% of all pregnancies in the US, though its frequency of use has been decreasing over the past decade and a half.

[0021] Normal humans have two sets of 23 chromosomes in every healthy, diploid cell, with one copy coming from each parent. Aneuploidy, a condition in a nuclear cell where the cell contains too many and/or too few chromosomes is believed to be responsible for a large percentage of failed implantations, miscarriages, and genetic diseases. Detection of chromosomal abnormalities can identify individuals or embryos with conditions such as Down syndrome, Klinefelter's syndrome, and Turner syndrome, among others, in addition to increasing the chances of a successful pregnancy. Testing for chromosomal abnormalities is especially important as the mother's age: between the ages of 35 and 40 it is estimated that at least 40% of the embryos are abnormal, and above the age of 40, more than half of the embryos are abnormal.

[0022] It has recently been discovered that cell-free fetal DNA and intact fetal cells can enter maternal blood circulation. Consequently, analysis of this genetic material can allow early

NPD. Improved methods are desired to improve the sensitivity and specificity and reduce the time and cost required for NPD.

SUMMARY OF THE INVENTION

[0023] In one aspect, the invention features methods of amplifying target loci in a nucleic acid sample. In some embodiments, the method involves (i) contacting the nucleic acid sample with a library of test primers (such as non-immobilized primers) that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture; and (ii) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products that include target amplicons. In some embodiments, the method also includes determining the presence or absence of at least one target amplicon (such as at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target amplicons). In some embodiments, the method also includes determining the sequence of at least one target amplicon (such as at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target amplicons). In some embodiments, the method involves multiplex PCR and sequencing (such as high throughput sequencing). In some embodiments, the method includes selecting the test primers from a library of candidate primers by removing one or more of the candidate primers based at least in part on the likelihood of dimer formation between candidate primers (such as ΔG values, undesirability scores, or interaction scores) prior to contacting the nucleic acid sample with the library of test primers.

[0024] In some embodiments, the method involves (i) contacting a sample comprising target human loci with a library of at least 50 (such as at least 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-immobilized, non-identical primers that simultaneously hybridize to at least 50 (such as at least 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-identical target human loci to produce a reaction mixture; wherein the primers do not include molecular inversion probes (MIPs); (ii) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein the annealing temperature for the reaction conditions is greater than a melting temperature (such as

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the empirically measured or calculated T_m) of at least 50 (such as at least 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) of the non-identical primers and/or the length of the annealing step of the reaction conditions is greater than 5 minutes (such as at least 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes; and wherein at least 50 (such as at least 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-identical target human loci are simultaneously amplified; and (iii) detecting the amplified products such as by sequencing the amplified products or hybridizing the amplified products to an array. In some embodiments, the method includes empirically measuring or calculating (such as calculating with a computer) the melting temperature of at least 25, 50, 80, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library and selecting an annealing temperature that satisfies any of these embodiments for PCR amplification of target loci.

[0025] In some embodiments, the method involves (i) contacting a sample comprising target human loci with a library of at least 2 (such as at least 5, 10, 25 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-immobilized, non-identical primers that simultaneously hybridize to at least 2 (such as at least 5, 10, 25 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-identical target human loci to produce a reaction mixture; wherein the primers do not include molecular inversion probes (MIPs); (ii) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein at least 2 (such as at least 5, 10, 25 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000) non-identical target human loci are simultaneously amplified; and (iii) detecting the amplified products such as by sequencing the amplified products or hybridizing the amplified products to an array. In various embodiments, (i) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 2, 5, 10, 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers; (ii) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3

to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 2, 5, 10, 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers; (iii) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers; (iv) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers; (v) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 2, 5, 10, 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers; or (vi) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 2, 5, 10, 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In various embodiments, (i) the length of the annealing step (per PCR cycle) is greater than 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes or (ii) the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive. In various embodiments, any of the embodiments for annealing temperature are combined with any of the embodiments for annealing time. In various embodiments, the annealing temperature is at least 3 °C greater than the melting temperature of at least 50 of the non-identical primers, the annealing temperature is at least 3 °C greater than the highest melting temperature of the primers, the annealing temperature is at least 8 °C greater than the highest melting temperature of the primers, the annealing temperature is at least 3 °C greater than the average melting temperature of the primers, the annealing temperature is at least 8 °C greater than the average melting temperature of the primers, the range of melting temperature of the primers is between 1 to 5 °C, inclusive, the range of melting temperatures of the primers is less than 5 °C, or any combination thereof. In some embodiments, the method includes empirically measuring or

calculating (such as calculating with a computer) the melting temperature of at least 25, 50, 80, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library and selecting an annealing temperature that satisfies any of these embodiments for PCR amplification of target loci. In some embodiments, a crowding agent, such as PEG or glycerol is included in the reaction mixture.

[0026] In various embodiments of any of the aspects of the invention, the method includes non-specifically amplifying nucleic acids in a sample comprising target human loci; contacting the amplified nucleic acids with a library of non-identical primers (such as non-immobilized primers) that simultaneously hybridize to at least 1,000 non-identical target human loci to produce a reaction mixture; wherein the primers do not include molecular inversion probes (MIPs); subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein at least 1,000 non-identical target human loci are simultaneously amplified; and sequencing the amplified products. In some embodiments, the non-specific amplification comprises universal polymerase chain reaction (PCR), whole genome application, ligation-mediated PCR, degenerate oligonucleotide primer PCR, or multiple displacement amplification. In some embodiments, the method includes contacting a sample comprising target human loci with a library of non-identical primers (such as non-immobilized primers) that simultaneously hybridize to at least 1,000 non-identical target human loci to produce a reaction mixture; wherein the primers do not include molecular inversion probes (MIPs); subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein the annealing temperature for the reaction conditions is greater than the melting temperature of at least 1,000 of the non-identical primers; and wherein at least 1,000 non-identical target human loci are simultaneously amplified; and sequencing the amplified products. In some embodiments, the method includes contacting a sample comprising target human loci with a library of non-identical primers (such as non-immobilized primers) that simultaneously hybridize to at least 1,000 non-identical target human loci to produce a reaction mixture in which the concentration of each primer is less than 20 nM; wherein the primers do not include molecular inversion probes (MIPs); subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein the length of the annealing step of the reaction conditions is greater than 10 minutes; and wherein at least 1,000 non-identical target human loci are simultaneously amplified; and sequencing the amplified

products. In some embodiments, the method includes contacting a sample comprising target human loci with a library of non-identical primers (such as non-immobilized primers) that simultaneously hybridize to at least 1,000 non-identical target human loci to produce a reaction mixture; wherein the guanine-cytosine (GC) content of the primers is between 30% and 80%, inclusive; wherein the range of melting temperatures of the primers is less than 5 °C; wherein the length of the primers is between 15 to 75 nucleotides, inclusive; and wherein the primers do not include molecular inversion probes (MIPs); subjecting the reaction mixture to primer extension reaction conditions to produce amplified products comprising target amplicons; wherein at least 1,000 non-identical target human loci are simultaneously amplified; and sequencing the amplified products. In some embodiments, the method does not comprise using a microarray. In some embodiments, the library includes a least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified.

[0027] In various embodiments of any of the aspects of the invention, the ΔG values for each possible combination of two primers in the library are all equal to or greater than -5 kcal/mol. In some embodiments, the method simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the sample using at least 1,000 non-identical primer pairs (such as non-immobilized primer pairs) to produce a first set of amplified products; wherein each primer pair includes a forward primer and a reverse primer that hybridize to the same target human locus. In some embodiments, the method also includes simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the first set of amplified products using at least 1,000 non-identical primer pairs (such as non-immobilized primer pairs) to produce a second set of amplified products; wherein each primer pair includes a forward primer and a reverse primer that hybridize to the same target human locus. In some embodiments, the primer pairs used in the first and second round of PCR are the same. In some embodiments, the primer pairs used in the first and second round of PCR are different. In some embodiments, the forward primers used in the first and second round of PCR are the same, and the reverse primers used in the first and second round of PCR are different. In some embodiments, the forward primers used in the first and second round of PCR are different, and the reverse

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primers used in the first and second round of PCR are the same. In some embodiments, the method simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the sample using at least 1,000 non-identical primer pairs (such as non-immobilized primer pairs) to produce a first set of amplified products; wherein each primer pair includes an outer forward primer and an outer reverse primer that hybridize to the same target human locus; and simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the first set of amplified products using a universal reverse primer and at least 1,000 non-identical inner forward primers to produce a second set of amplified products; wherein each inner forward primer hybridizes to a region downstream from the corresponding outer forward primer. In some embodiments, the method includes simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the sample using at least 1,000 non-identical primer pairs to produce a first set of amplified products; wherein each primer pair includes an outer forward primer and an outer reverse primer that hybridize to the same target human locus; and simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the first set of amplified products using a universal forward primer and at least 1,000 non-identical inner reverse primers to produce a second set of amplified products; wherein each inner reverse primer hybridizes to a region upstream from the corresponding outer reverse primer. In some embodiments, the method includes simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the sample using at least 1,000 non-identical forward primers and a universal reverse primer to produce a first set of amplified products; and simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the first set of amplified products using a universal forward primer and at least 1,000 non-identical reverse primers to produce a second set of amplified products. In some embodiments, the method includes simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the sample using at least 1,000 non-identical reverse primers and a universal forward primer to produce a first set of amplified products; and simultaneously PCR-amplifying at least 1,000 non-identical target human loci in the first set of amplified products using a universal reverse primer and at least 1,000 non-identical forward primers to produce a second set of amplified products. In some embodiments, at least 96% of the primer molecules are extended to form amplified products. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000;

30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature of the non-identical primers. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In some embodiments, the range of melting temperature of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers is between 1 to 5 °C, inclusive. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers have 2, 1, or 0 guanines or cytosines in the last 5 bases at the 3' end of the primers. In some embodiments, the sample comprises maternal DNA from the pregnant mother of a fetus and fetal DNA, and wherein the method comprises determining the presence or absence of a fetal chromosome abnormality from the sequencing data. In some embodiments, the sample is from an individual suspected of having cancer or an above normal risk for cancer; and wherein one or more of the target human loci comprises a polymorphism or other mutation associated with an above normal risk for cancer or associated with cancer.

[0028] In various embodiments of any of the aspects of the invention, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the amplified products are target amplicons. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In various embodiments, less than 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers. In some embodiments, the library of test primers includes at least 25; 50; 75; 100; 300; 500; 750;

1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 test primer pairs, wherein each pair of primers includes a forward test primer and a reverse test primer that hybridize to the same target locus. In some embodiments, the library of test primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 individual test primers that hybridize to different target loci, wherein the individual primers are not part of primer pairs.

[0029] In various embodiments of any of the aspects of the invention, the concentration of each test primer is less than 100, 75, 50, 25, 10, 5, 2, 1, 0.5, 0.1, or 0.05 nM. In various embodiments, the guanine-cytosine (GC) content of the test primers is between 30 to 80%, such as between 20 to 70%, 40 to 70%, or 50 to 60%, inclusive. In some embodiments, the range of GC content (*e.g.,* the maximum GC content minus minimum GC content, such as 80% - 60% = a range of 20%) of the test primers is less than 30, 20, 10, or 5%. In some embodiments, there are at least 2 (such as 3, 4, or 5) guanines or cytosines in the last 5 bases at the 3' end of the primers. In some embodiments, a maximum of 2 (such as 2, 1, or 0) of the bases in the last 5 bases at the 3' end of the primers are guanines or cytosines. In some embodiments, there are at least 1 (such as 2 or 3) guanines or cytosines in the last 3 bases at the 3' end of the primers. In some embodiments, the melting temperature (T_m) of the test primers is between 40 to 80 °C, such as 50 to 70 °C, 55 to 65 °C, 54 to 60.5 °C, or 57 to 60.5 °C, inclusive. In some embodiments, the range of melting temperatures of the test primers is less than 20, 15, 10, 5, 3, or 1 °C. In some embodiments, the length of the test primers is between 15 to 100 nucleotides, such as between 15 to 75 nucleotides, 15 to 40 nucleotides, 17 to 35 nucleotides, 18 to 30 nucleotides, or 20 to 65 nucleotides, inclusive. In some embodiments, the test primers include a tag that is not target specific, such as a tag that forms an internal loop structure. In some embodiments, the tag is between two DNA binding regions. In various embodiments, the test primers include a 5' region that is specific for a target locus, an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. In various embodiments, the length of the 3' region is at least 7 nucleotides. In some embodiments, the length of the 3' region is between 7 and 20 nucleotides, such as between 7 to 15 nucleotides, or 7 to 10 nucleotides, inclusive. In various embodiments, the test primers include a 5' region that is not specific for a target locus (such as a tag or a universal primer binding site) followed by a

region that is specific for a target locus, an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. In some embodiments, the range of the length of the test primers is less than 50, 40, 30, 20, 10, or 5 nucleotides. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides, inclusive. In some embodiments, the length of the target amplicons is at least 100; 200; 300; 400; 500; 600; 700; 800; 900; 1,000; 1,200; 1,500; 2,000; or 3,000 nucleotides. In some embodiments, the length of the target amplicons is between 100 and 1,500 nucleotides, such as between 100 to 1,000; 100 to 500, 500 to 750, or 750 to 1,000 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 or all of the target amplicons have a length that falls within the range of the average length of the amplicons ± 5% of the average length, average length ± 20%, average length ± 20%, average length ± 30%, or average length ± 50%.

[0030] In various embodiments of any of the aspects of the invention, the primer extension reaction conditions are polymerase chain reaction conditions (PCR). In various embodiments, the length of the annealing step is greater than 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes. In various embodiments, the length of the extension step is greater than 0.2, 0.5, 1, 3, 5, 8, 10, or 15 minutes.

[0031] In various embodiments of any of the aspects of the invention, the test primers are used to simultaneously amplify at least 300 different target loci in a sample that includes maternal DNA from the pregnant mother of a fetus and fetal DNA to determine the presence or absence of a fetal chromosome abnormality. In various embodiments, the method includes ligating a universal primer binding site to the DNA molecules in the sample; amplifying the ligated DNA molecules using at least 300 specific primers and a universal primer to produce a first set of amplified products; and amplifying the first set of amplified products using at least 300 pairs of specific primers to produce a second set of amplified products.

[0032] In various embodiments of any of the aspects of the invention, the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in sample includes DNA from an alleged father of a fetus and to simultaneously amplify the

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target loci in a sample that includes maternal DNA from the pregnant mother of the fetus and fetal DNA to establish whether the alleged father is the biological father of the fetus.

[0033] In various embodiments of any of the aspects of the invention, the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in one cell or multiple cells from an embryo to determine the presence or absence of a chromosome abnormality. In various embodiments, cells from a set of two or more embryos are analyzed, and one embryo is selected for *in vitro* fertilization.

[0034] In various embodiments of any of the aspects of the invention, the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a forensic nucleic acid sample. In various embodiments, the length of the annealing step is greater than 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes

[0035] In various embodiments of any of the aspects of the invention, the method involves using the test primers to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a control nucleic acid sample to produce a first set of target amplicons and to simultaneously amplify the target loci in a test nucleic acid sample to produce a second set of target amplicons; and comparing the first and second sets of target amplicons to determine whether a target locus is present in one sample but absent in the other, or whether a target locus is present at different levels in the control sample and the test sample. In various embodiments, the test sample is from an individual suspected of having a disease or phenotype of interest (such as cancer), or an increased risk (such as an above normal level of risk) for a disease or phenotype of interest; and wherein one or more of the target loci include a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased risk (such as an above normal level of risk) for the disease or phenotype of interest, or associated with the disease or phenotype of interest. In various embodiments, the method involves using the test primers to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a control sample that includes RNA to produce a first set of target amplicons and to simultaneously amplify the target loci in a test sample that includes RNA to produce a second set of target amplicons; and comparing the first and second sets of target amplicons to determine the presence or absence of a difference in the RNA expression levels between the control sample and the test sample. In various embodiments, the RNA is mRNA. In various embodiments, the test sample is from an individual suspected of having a disease or phenotype of interest (such as cancer) or an increased risk for the disease or phenotype of interest (such as

cancer); and wherein one or more of the target loci includes a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased risk for the disease or phenotype of interest or associated with the disease or phenotype of interest. In some embodiments, the test sample is from an individual diagnosed with a disease or phenotype of interest (such as cancer); and wherein a difference in the RNA expression level between the control sample and test sample indicates a target locus includes a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased or decreased risk for the disease or phenotype of interest.

[0036] In some embodiments of any of the aspects of the invention, the test primers are selected from a library of candidate primers based on one or more parameters, such as the selection of primers using any of the methods of the invention. In some embodiments, the test primers are selected from a library of candidate primers based at least in part on the ability of the candidate primers to form primer dimers.

[0037] In one aspect, the invention features methods of selecting test primers from a library of candidate primers. In various embodiments, the selection involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing the candidate primer with the highest score (such as an undesirability score) from the library of candidate primers; and (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a library of test primers. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below a minimum threshold. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number. In various embodiments, a score (such as an undesirability score) is calculated for at least 80, 90, 95, 98, 99, or 99.5% of the possible combinations of candidate primers in the library. In various embodiments, the candidate primers remaining in the library are capable of simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In various embodiments, the method also includes (v) contacting a nucleic acid sample that includes target loci with the candidate primers remaining

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in the library to produce a reaction mixture; and (vi) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products that includes target amplicons.

[0038] In one aspect, the invention features methods of selecting test primers from a library of candidate primers. In various embodiments, the selection of test primers are selected from a library of candidate primers involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing from the library of candidate primers the candidate primer that is part of the greatest number of combinations of two candidate primers with a score (such as an undesirability score) above a first minimum threshold; (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a library of test primers. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the first minimum threshold. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number. In various embodiments, a score (such as an undesirability score) is calculated for at least 80, 90, 95, 98, 99, or 99.5% of the possible combinations of candidate primers in the library. In various embodiments, the candidate primers remaining in the library are capable of simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In various embodiments, the method also includes (v) contacting a nucleic acid sample that includes target loci with the candidate primers remaining in the library to produce a reaction mixture; and (vi) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products that includes target amplicons.

[0039] In various embodiments of any of the aspects of the invention, the selection method involves further reducing the number of candidate primers remaining in the library by decreasing the first minimum threshold used in step (ii) to a lower second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection method involves increasing the first minimum threshold used in step (ii) to a higher second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection

method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the second minimum threshold, or until the number of candidate primers remaining in the library is reduced to a desired number.

[0040] In various embodiments of any of the aspects of the invention, the method involves, prior to step (i), identifying or selecting primers that hybridize to the target loci. In some embodiments, multiple primers (or primer pairs) hybridize to the same target locus, and the selection method is used to select a one primer (or one primer pair) for this target locus based on one or more parameters. In various embodiments, the method involves, prior to step (ii), removing a primer pair from the library that produces a target amplicon that overlaps with a target amplicon produced by another primer pair. In various embodiments, a candidate primer is selected out of a group of two or more candidate primers with equal scores (such as undesirability scores) for removal from the library of candidate primers based on one or more other parameters. In some embodiments, the candidate primers remaining in the library are used as a library of test primers in any of the methods of the invention. In some embodiments, the resulting library of test primers includes any of the primer libraries of the invention.

[0041] In various embodiments of any of the aspects of the invention, the selection method selects candidate primers and divides them into different pools (*e.g.*, 2, 3, 4, 5, 6, or more different pools). Each pool can be used to simultaneously amplify a large number of target loci (or a subset of target loci) in a single reaction volume. In some embodiments, a graph coloring algorithm is used to divide candidate primers into different pools. If desired, this method can be used to minimize the number of different pools needed to amplify most or all of the target loci.

[0042] In some embodiments, most or all of the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the target loci) are amplified by at least 2, 3, 4, 5, 6, or more different pools. In some embodiments, most or all of the bases in the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the bases in the target loci) are amplified by at least 2,3, 4, 5, 6, or more different pools. In some embodiments, most or all of the bases in the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the bases in the target loci) are amplified by at least 2, 3, 4, 5, 6, or more different primers or primer pairs in different pools. For example, a particular base in a target locus may be amplified by at least 2, 3, 4, 5, 6, or more different primers or primer pairs; wherein each different primer or primer pair is in a

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different pool. Using different primers or primer pairs to amplify each base allows multiple independent measurements of the base to be made, thereby increasing the accuracy of the method. Dividing the different primers or primer pairs that amplify the same base into different pools prevents interference due to overlapping amplicons being formed by different primers or primer pairs.

[0043] In one aspect, the invention features methods of selecting test primers from a library of candidate primers to form 2 or more different primer pools. In various embodiments, the selection involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing the candidate primer with the highest score (such as an undesirability score) from the library of candidate primers; and (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a first pool. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below a minimum threshold for the first pool. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In some embodiments, after the first pool is selected those primers are removed from further consideration and steps of the method (such as steps (ii) and (iii)) are repeated with the remaining primers to select a second pool. If desired, this method may be repeated to select the desired number of primer pools. In various embodiments, a score (such as an undesirability score) is calculated for at least 80, 90, 95, 98, 99, or 99.5% of the possible combinations of candidate primers in the library. In some embodiments, the score is based at least in part on the current coverage of the bases in the target locus (such as the number of other primer pools that have a primer or primer pair that amplifies a particular base in the target locus). In various embodiments, one or more of the pools are each capable of simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In various embodiments, the method also includes separately contacting a nucleic acid sample that includes target loci with two or more of the pools to

produce separate reaction mixtures; and (vi) subjecting the reaction mixtures to primer extension reaction conditions to produce amplified products that includes target amplicons.

[0044] In one aspect, the invention features methods of selecting test primers from a library of candidate primers to form 2 or more different primer pools. In various embodiments, the selection of test primers are selected from a library of candidate primers involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing from the library of candidate primers the candidate primer that is part of the greatest number of combinations of two candidate primers with a score (such as an undesirability score) above a first minimum threshold; (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a first pool. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the first minimum threshold for the first pool. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In various embodiments, the selection method involves further reducing the number of candidate primers remaining in the library by decreasing the first minimum threshold used in step (ii) to a lower second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection method involves increasing the first minimum threshold used in step (ii) to a higher second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the second minimum threshold, or until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In some embodiments, after the first pool is selected those primers are removed from further consideration and steps of the method (such as steps (ii) and (iii)) are repeated with the remaining primers to select a second pool. If desired, this method may be repeated to select the desired number of primer pools. In various embodiments, a score (such as an undesirability score) is calculated for at least 80, 90, 95, 98, 99, or 99.5% of the possible

combinations of candidate primers in the library. In some embodiments, the score is based at least in part on the current coverage of the bases in the target locus (such as the number of other primer pools that have a primer or primer pair that amplifies a particular base in the target locus). In various embodiments, one or more of the pools are each capable of simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In various embodiments, the method also includes separately contacting a nucleic acid sample that includes target loci with two or more of the pools to produce separate reaction mixtures; and (vi) subjecting the reaction mixtures to primer extension reaction conditions to produce amplified products that includes target amplicons.

[0045] In some embodiments, at least 70, 80, 85, 90, 95, or 100% of the nucleotides in a region of interest (such as an exon) are included in at least 1, 2, 3, or 4 different amplicons (*i.e.*, amplicons with non-identical sequences that are formed by different primers or primer pairs). In some embodiments, at least 70, 80, 85, 90, 95, or 100% of the nucleotides in at least 70, 80, 85, 90, 95, or 100% of the regions of interest are amplified by at least 1, 2, 3, or 4 different amplicons. In some embodiments in which 2-fold coverage is desired, the primers are divided into at least two different pools such the amplicons in each pool do not overlap with each other (which would cause interference during amplification).

[0046] In various embodiments of any of the aspects of the invention, the score (such as the undesirability score) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, disease prevalence associated with a sequence (*e.g.*, a polymorphism) at the target locus, disease penetrance associated with a sequence (*e.g.*, a polymorphism) at the target locus, specificity of the candidate primer for the target locus, size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon.

[0047] In various embodiments of any of the aspects of the invention, the score (such as the undesirability score) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, specificity of the candidate primer for the target locus; size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of

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the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon; and the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a sample that includes maternal DNA from the pregnant mother of a fetus and fetal DNA to determine the presence or absence of a fetal chromosome abnormality. In various embodiments, the method includes ligating a universal primer binding site to the DNA molecules in the sample; amplifying the ligated DNA molecules using *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) specific primers and a universal primer to produce a first set of amplified products; and amplifying the first set of amplified products using *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) pairs of specific primers to produce a second set of amplified products. In various embodiments, at least 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primer pairs are used. In various embodiments, at least 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0048] In various embodiments of any of the aspects of the invention, the score (such as the undesirability score) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, specificity of the candidate primer for the target locus; size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon; and the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in sample includes DNA from an alleged father of a fetus and to simultaneously amplify the target loci in a sample that includes maternal DNA from the pregnant mother of a fetus and fetal DNA to establish whether the alleged father is the biological father of the fetus. In various embodiments, at least 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000;

20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0049] In various embodiments of any of the aspects of the invention, the score (such as the undesirability score) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, specificity of the candidate primer for the target locus; size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon; and the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in one cell or multiple cells from an embryo to determine the presence or absence of a chromosome abnormality. In various embodiments, cells from a set of two or more embryos are analyzed, and one embryo is selected for *in vitro* fertilization. In various embodiments, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0050] In various embodiments of any of the aspects of the invention, the scores (such as the undesirability scores) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, specificity of the candidate primer for the target locus; size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification

efficiency of the target amplicon, and size of the target amplicon; and the test primers are used to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a forensic nucleic acid sample. In various embodiments, the length of the annealing step is greater than 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes. In various embodiments, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0051] In various embodiments of any of the aspects of the invention, the scores (such as the undesirability scores) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, disease prevalence associated with a sequence (*e.g.*, a polymorphism) at the target locus, disease penetrance associated with a sequence (*e.g.*, a polymorphism) at the target locus, specificity of the candidate primer for the target locus, size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon; and the method involves using the test primers to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000) different target loci in a control nucleic acid sample to produce a first set of target amplicons and to simultaneously amplify the target loci in a test nucleic acid sample to produce a second set of target amplicons; and comparing the first and second sets of target amplicons to determine whether a target locus is present in one sample but absent in the other, or whether a target locus is present at different levels in the control sample and the test sample. In various embodiments, the test sample is from an individual suspected of having a disease or phenotype of interest, or an increased risk for a disease or phenotype of interest; and wherein one or more of the target loci include a sequence (*e.g.*, a polymorphism) at the target locus associated with an increased risk for the disease or phenotype of interest, or associated with the disease or phenotype of interest. In various embodiments, at least 300, 500; 750; 1,000; 2,000; 5,000;

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7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0052] In various embodiments of any of the aspects of the invention, the scores (such as the undesirability scores) are based at least in part on one or more parameters selected from the group consisting of heterozygosity rate of the target locus, disease prevalence associated with a sequence (*e.g.*, a polymorphism) at the target locus, disease penetrance associated with a sequence (*e.g.*, a polymorphism) at the target locus, specificity of the candidate primer for the target locus, size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, and size of the target amplicon; and the method involves using the test primers to simultaneously amplify *e.g.*at least 100 (*e.g.*, at least 300 or 1,000); different target loci in a control sample that includes RNA to produce a first set of target amplicons and to simultaneously amplify the target loci in a test sample that includes RNA to produce a second set of target amplicons; and comparing the first and second sets of target amplicons to determine the presence or absence of a difference in the RNA expression levels between the control sample and the test sample. In various embodiments, the RNA is mRNA. In various embodiments, the test sample is from an individual suspected of having a disease or phenotype of interest (such as cancer) or an increased risk for the disease or phenotype of interest (such as cancer); and wherein one or more of the target loci includes a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased risk for the disease or phenotype of interest or associated with the disease or phenotype of interest. In some embodiments, the test sample is from an individual diagnosed with a disease or phenotype of interest (such as cancer); and wherein a difference in the RNA expression level between the control sample and test sample indicates a target locus includes a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased or decreased risk for the disease or phenotype of interest. In various embodiments, at least 300, 500; 750; 1,000; 2000; 5,000;

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7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold.

[0053] In one aspect, the invention features libraries of primers (such as non-immobilized primers). In some embodiments, the primers are selected from a library of candidate primers using any of the methods of the invention. In some embodiments, the library includes primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In some embodiments, the library includes primers that simultaneously amplify at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci. In some embodiments, the library includes primers that simultaneously amplify at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci such that less than 60, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers. In some embodiments, the library includes primers that simultaneously amplify at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci such that at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the amplified products are target amplicons. In some embodiments, the library includes primers that simultaneously amplify target loci such that at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci out of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target

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loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers or primer pairs. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 primer pairs, wherein each pair of primers includes a forward test primer and a reverse test primer where each pair of test primers hybridize to a target locus. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 individual primers that each hybridize to a different target locus, wherein the individual primers are not part of primer pairs. In some embodiments, the primers in the library are not immobilized (such as not immobilized to a solid support) or not part of a microarray. In some embodiments, the primers are dissolved in solution (such as dissolved in the liquid phase). In some embodiments, the library of primers consists essentially of, or consists of primers.

[0054] In some embodiments, ΔG values for each possible combination of two primers (each possible primer dimer) in a library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol. In some embodiments, ΔG values for at least 80, 85, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library for possible combinations of that primer with other primers in the library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol.

[0055] In various embodiments of any of the aspects of the invention, the concentration of each primer is less than 100, 75, 50, 25, 10, 5, 2, 1, 0.5, 0.1, or 0.05 nM. In various embodiments, the GC content of the primers is between 30 to 80%, such as between 40 to 70%, 20 to 70%, or 50 to 60%, inclusive. In some embodiments, the range of GC content of the primers is less than 30, 20, 10, or 5%. In some embodiments, there are at least 2 (such as 3, 4, or 5) guanines or cytosines in the last 5 bases at the 3' end of the primers. In some embodiments, there are at least 1 (such as 2 or 3) guanines or cytosines in the last 3 bases at the 3' end of the primers. In some embodiments, a maximum of 2 (such as 2, 1, or 0) bases in the last 5 bases at the 3' end of the primers are guanines or cytosines. In some embodiments, the melting temperature of the primers is between 40 to 80 °C, such as 50 to 70 °C, 55 to 65

°C, 54 to 60.5 °C, or 57 to 60.5 °C, inclusive. In some embodiments, the range of melting temperature of the primers is less than 15, 10, 5, 3, or 1 °C. In some embodiments, the length of the primers is between 15 to 100 nucleotides, such as between 15 to 75 nucleotides, 15 to 40 nucleotides, 17 to 35 nucleotides, 18 to 30 nucleotides, or 20 to 65 nucleotides, inclusive. In some embodiments, the primers include a tag that is not target specific, such as a tag that forms an internal loop structure. In some embodiments, the tag is between two DNA binding regions. In various embodiments, the primers include a 5' region that is specific for a target locus, an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. In various embodiments, the length of the 3' region is at least 7 nucleotides. In some embodiments, the length of the 3' region is between 7 and 20 nucleotides, such as between 7 to 15 nucleotides, or 7 to 10 nucleotides, inclusive. In various embodiments, the primers include a 5' region that is not specific for a target locus (such as another tag or a universal primer binding site) followed by a region that is specific for a target locus**,** an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. In some embodiments, the range of the length of the primers is less than 50, 40, 30, 20, 10, or 5 nucleotides. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the target loci are on two or more different chromosomes, such as two or more of chromosomes 13, 18, 21, X and Y. In some embodiments, the target loci are target human loci. In some embodiments, the target loci includes a sequence (*e.g.*, a polymorphism or other mutation) associated with an increased risk for the disease or phenotype of interest (such as cancer), or associated with the disease or phenotype of interest (such as cancer). In some embodiments, the polymorphism or mutation is a driver mutation that has a causative role in the disease or phenotype of interest (such as cancer). In some embodiments, the polymorphism or mutation is not a causative mutation. For example, in some cancers, multiple mutations accumulate but some of them are not causative mutations. Polymorphisms or mutations (such as those that are present at a higher frequency in subjects with a disease or phenotype of interest such as cancer than subjects without the disease or phenotype of interest such as cancer) that are not causative can still be useful for diagnosing the disease or phenotype. In some embodiments, the polymorphisms or mutation is present at a higher

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frequency in subjects with a disease or disorder (such as cancer) than subjects (such as healthy or normal subjects) without the disease or disorder (such as cancer). In some embodiments, the polymorphisms or mutation is indicative of cancer, such as a causative mutation. In some embodiments, the polymorphism(s) or mutation(s) are directly detected. In some embodiments, the polymorphism(s) or mutation(s) are indirectly detected by detection of one or more sequences (*e.g.*, a polymorphic locus such as a SNP) that are linked to the polymorphism or mutation).

[0056] In one aspect, the invention provides a composition that includes any of the primer libraries of the invention (such as non-immobilized primers). In some embodiments, the composition includes one or more free nucleotides (such as deoxynucleotides, ATP, CTP, GTP, TTP, UTP, dATP, dCTP, dGTP, dTTP, dUTP an activated nucleotide or deoxynucleotide, or a non-naturally occurring nucleotide or deoxynucleotide). In some embodiments, the composition includes at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with a polynucleotide sequence of a human nucleic acid and at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with a polynucleotide sequence that is not found in a human (such as a universal primer, a primer that comprises a region or consists entirely of random nucleotides, or a primer with a region such as a tag or barcode of one or more nucleotides that are not found in a human or are not found in nature as part of the polynucleotide sequence of the primer). In some embodiments, the composition includes at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with the polynucleotide sequence of a human nucleic acid and one or more non-human or non-naturally occurring enzymes (*e.g.*, ligase or polymerase from a species other than a human, such as a bacterial or non-naturally-occurring ligase or polymerase). In some embodiments, the composition includes at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with the polynucleotide sequence of a human nucleic acid and a buffer or additive that is non-naturally-occurring or is not found in a human. In some embodiments, the

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composition comprises, consists essentially of, or consists of one or more of the following: primer(s), amplicon(s) free nucleotide(s), non-human or non-naturally occurring enzyme(s), buffer(s), additive(s), or any combination thereof. In some embodiments, the composition comprises, consists essentially of, or consists of primers and one or more non-human or non-naturally occurring enzymes. Exemplary non-naturally occurring enzymes contain at least one sequence difference compared to naturally occurring (wild-type) enzymes.

[0057] In one aspect, the invention provides a composition comprising at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical amplicons) in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of at least 100 different target loci (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical target loci) using at least 100 different primers or primer pairs (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers or primer pairs) in one reaction volume. In some embodiments, (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 and 75 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, the composition includes at least 1,000 different amplicons in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of at least 1,000 different target human loci using at least 1,000 different primers in one reaction volume;

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wherein (i) less than 20% of the amplicons are primer dimers, and (ii) at least 80% of the amplicons comprise one of the target human loci and are between 50 and 100 nucleotides in length, inclusive. In some embodiments, the composition consists essentially of, or consists of one or more of the following: amplicons, primers (such as any of the primers disclosed herein), free nucleotide(s), non-human or non-naturally occurring enzyme(s), buffer(s), or any combination thereof. In some embodiments, at least one amplicon or primer has a non-human or non-naturally occurring sequence, nucleotide, or linkage between nucleotides.

[0058] In one aspect, the invention provides a composition comprising at least 100 different primers or primer pairs (*e.g.*, at least 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers or primer pairs) and at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical amplicons) in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of at least 100 different target loci (*e.g.*, at least 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical target loci) using the primers or primer pairs in one reaction volume. In some embodiments, (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 and 75 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, the composition comprising at least 1,000 different primers and at least 1,000 different amplicons in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of

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at least 1,000 different target human loci with the primers in one reaction volume; wherein (i) less than 20% of the amplicons are primer dimers, and (ii) at least 80% of the amplicons comprise one of the target loci and are between 50 and 100 nucleotides in length, inclusive. In some embodiments, the composition consists essentially of, or consists of one or more of the following: amplicons, primers (such as any of the primers disclosed herein), free nucleotide(s), non-human or non-naturally occurring enzyme(s), buffer(s), or any combination thereof. In some embodiments, at least one amplicon or primer has a non-human or non-naturally occurring sequence, nucleotide, or linkage between nucleotides.

[0059] In one aspect, the invention provides kits that include any of the primer libraries or compositions of the invention for amplifying target loci in a nucleic acid sample. In some embodiments, the kits consists essentially of, or consists of primers, primers and instructions for using the primers, a composition of the invention, or a composition of the invention and instructions for using the composition. In some embodiments, the kit includes instructions for using the library to amplify the target loci.

[0060] In one aspect, the invention provides an apparatus, device, or composition that includes any of the primer libraries or compositions of the invention. In some embodiments, the apparatus, device, or composition includes a physical structure (such as one or more reaction vessels, reaction chambers, or wells) that contains the primer library or composition of the invention (for example, the primers may be dissolved in a solution that is in the physical structure). In some embodiments, the physical structure is a non-naturally occurring physical structure or a physical structure that does not naturally contain a primer library or composition of the invention (such as a physical structure that is not found in nature with nucleic acids in it).

[0061] In one aspect, the invention features methods for determining a ploidy status of chromosome in a gestating fetus. In some embodiments, the method involves contacting a nucleic acid sample with a library of primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different polymorphic loci to produce a reaction mixture; wherein the nucleic acid sample includes maternal DNA from the mother of the fetus and fetal DNA from the fetus. In some embodiments, the reaction mixture is subjected to primer extension reaction conditions to produce amplified products; the amplified products are measured with a high throughput sequencer to produce sequencing data;

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allele counts at the polymorphic loci are calculated on a computer based on the sequencing data; a plurality of ploidy hypotheses each pertaining to a different possible ploidy state of the chromosome are created on a computer; a joint distribution model for the expected allele counts at the polymorphic loci on the chromosome is built on a computer for each ploidy hypothesis; a relative probability of each of the ploidy hypotheses is determined on a computer using the joint distribution model and the allele counts; and the ploidy state of the fetus is called by selecting the ploidy state corresponding to the hypothesis with the greatest probability.

[0062] In one aspect, the invention features methods for determining a ploidy status of a chromosome in a gestating fetus. In an embodiment a method for determining a ploidy status of a chromosome in a gestating fetus includes obtaining a first sample of DNA that comprises maternal DNA from the mother of the fetus and fetal DNA from the fetus, preparing the first sample by isolating the DNA so as to obtain a prepared sample, measuring the DNA in the prepared sample at a plurality of polymorphic loci on the chromosome, calculating, on a computer, allele counts at the plurality of polymorphic loci from the DNA measurements made on the prepared sample, creating, on a computer, a plurality of ploidy hypotheses each pertaining to a different possible ploidy state of the chromosome, building, on a computer, a joint distribution model for the expected allele counts at the plurality of polymorphic loci on the chromosome for each ploidy hypothesis, determining, on a computer, a relative probability of each of the ploidy hypotheses using the joint distribution model and the allele counts measured on the prepared sample, and calling the ploidy state of the fetus by selecting the ploidy state corresponding to the hypothesis with the greatest probability.

[0063] In one aspect, the invention features methods of testing for an abnormal distribution of a chromosome in a sample that includes a mixture of maternal and fetal DNA. In some embodiments, the method involves (i) contacting the sample with a library of primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture; wherein the target loci are from a plurality of different chromosomes; and wherein the plurality of different chromosomes include at least one first chromosome suspected of having an abnormal distribution in the sample and at least one second chromosome presumed to be normally distributed in the sample; (ii) subjecting the reaction mixture to primer extension reaction conditions to produce

amplified products; (iii) sequencing the amplified products to obtain a plurality of sequence tags aligning to the target loci; wherein the sequence tags are of sufficient length to be assigned to a specific target locus; (iv) assigning on a computer the plurality of sequence tags to their corresponding target loci; (v) determining on a computer a number of sequence tags aligning to the target loci of the first chromosome and a number of sequence tags aligning to the target loci of the second chromosome; and (vi) comparing on a computer the numbers from step (v) to determine the presence or absence of an abnormal distribution of the first chromosome.

[0064] In one aspect, the invention provides methods for detecting the presence or absence of a fetal aneuploidy. In some embodiments, the method involves (i) contacting a sample that includes a mixture of maternal and fetal DNA with a library of primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different non-polymorphic target loci to produce a reaction mixture; wherein the target loci are from a plurality of different chromosomes; (ii) subjecting the reaction mixture to primer extension reaction conditions to produce amplified products that includes target amplicons; (iii) quantifying on a computer a relative frequency of the target amplicons from the first and second chromosomes of interest; (iv) comparing on a computer the relative frequency of the target amplicons from the first and second chromosomes of interest; and (v) identifying the presence or absence of an aneuploidy based on the compared relative frequencies of the first and second chromosome of interest. In some embodiments, the first chromosome is a chromosome suspected of being euploid. In some embodiments, the second chromosome is a chromosome suspected of being aneuploidy.

[0065] In one aspect, a method is disclosed for determining presence or absence of fetal aneuploidy in a maternal tissue sample comprising fetal and maternal genomic DNA, the method including (a) obtaining a mixture of fetal and maternal genomic DNA from said maternal tissue sample, (b) conducting massively parallel DNA sequencing of DNA fragments randomly selected from the mixture of fetal and maternal genomic DNA of step (a) to determine the sequence of said DNA fragments, (c) identifying chromosomes to which the sequences obtained in step (b) belong, (d) using the data of step (c) to determine an amount of at least one first chromosome in said mixture of maternal and fetal genomic DNA, wherein said at least one first chromosome is presumed to be euploid in the fetus, (e) using the data of step (c) to determine an amount of a second chromosome in said mixture of maternal and fetal

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genomic DNA, wherein said second chromosome is suspected to be aneuploid in the fetus, (f) calculating the fraction of fetal DNA in the mixture of fetal and maternal DNA, (g) calculating an expected distribution of the amount of the second target chromosome if the second target chromosome is euploid, using the number in step (d), (h) calculating an expected distribution of the amount of the second target chromosome if the second target chromosome is aneuploid, using the first number is step (d) and the calculated fraction of fetal DNA in the mixture of fetal and maternal DNA in step (f), and (i) using a maximum likelihood or maximum a posteriori approach to determine whether the amount of the second chromosome as determined in step (e) is more likely to be part of the distribution calculated in step (g) or the distribution calculated in step (h); thereby indicating the presence or absence of a fetal aneuploidy.

[0066] In various embodiments of any of the aspects of the invention, the target loci include one or more SNPs in the homologous non-recombining region of chromosome X and/or chromosome Y. In some embodiments, the method includes determining the relative amount of chromosome X and chromosome Y. In some embodiments, the method includes determining the number of copies of chromosome X and/or chromosome Y.

[0067] In some embodiments, the method also includes obtaining genotypic data from one or both parents of the fetus. In some embodiments, obtaining genotypic data from one or both parents of the fetus includes preparing the DNA from the parents where the preparing comprises preferentially enriching the DNA at the plurality of polymorphic loci to give prepared parental DNA, optionally amplifying the prepared parental DNA, and measuring the parental DNA in the prepared sample at the plurality of polymorphic loci.

[0068] In various embodiments of any of the aspects of the invention, building a joint distribution model for the expected allele count probabilities of the plurality of polymorphic loci on the chromosome is done using the obtained genetic data from the one or both parents. In some embodiments, the sample (*e.g.*, the first sample) has been isolated from maternal plasma and where the obtaining genotypic data from the mother is done by estimating the maternal genotypic data from the DNA measurements made on the prepared sample.

[0069] In one aspect, a diagnostic box is disclosed for helping to determine a ploidy status of a chromosome in a gestating fetus where the diagnostic box is capable of executing the preparing and measuring steps of any of the methods of the invention.

[0070] In various embodiments of any of the aspects of the invention, the allele counts are probabilistic rather than binary. In some embodiments, measurements of the DNA in the

prepared sample at the plurality of polymorphic loci are also used to determine whether or not the fetus has inherited one or a plurality of disease linked haplotypes.

[0071] In various embodiments of any of the aspects of the invention, building a joint distribution model for allele count probabilities is done by using data about the probability of chromosomes crossing over at different locations in a chromosome to model dependence between polymorphic alleles on the chromosome. In some embodiments, building a joint distribution model for allele counts and the step of determining the relative probability of each hypothesis are done using a method that does not require the use of a reference chromosome.

[0072] In various embodiments of any of the aspects of the invention, determining the relative probability of each hypothesis makes use of an estimated fraction of fetal DNA in the prepared sample. In some embodiments, the DNA measurements from the prepared sample used in calculating allele count probabilities and determining the relative probability of each hypothesis comprise primary genetic data. In some embodiments, selecting the ploidy state corresponding to the hypothesis with the greatest probability is carried out using maximum likelihood estimates or maximum a posteriori estimates.

[0073] In various embodiments of any of the aspects of the invention, calling the ploidy state of the fetus also includes combining the relative probabilities of each of the ploidy hypotheses determined using the joint distribution model and the allele count probabilities with relative probabilities of each of the ploidy hypotheses that are calculated using statistical techniques taken from a group consisting of a read count analysis, comparing heterozygosity rates, a statistic that is only available when parental genetic information is used, the probability of normalized genotype signals for certain parent contexts, a statistic that is calculated using an estimated fetal fraction of the sample (*e.g.*, the first sample) or the prepared sample, and combinations thereof.

[0074] In various embodiments of any of the aspects of the invention, a confidence estimate is calculated for the called ploidy state. In some embodiments, the method also includes taking a clinical action based on the called ploidy state of the fetus, wherein the clinical action is selected from one of terminating the pregnancy or maintaining the pregnancy.

[0075] In various embodiments of any of the aspects of the invention, the method may be performed for fetuses at between 4 and 5 weeks gestation; between 5 and 6 weeks gestation; between 6 and 7 weeks gestation; between 7 and 8 weeks gestation; between 8 and 9 weeks gestation; between 9 and 10 weeks gestation; between 10 and 12 weeks gestation; between 12

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and 14 weeks gestation; between 14 and 20 weeks gestation; between 20 and 40 weeks gestation; in the first trimester; in the second trimester; in the third trimester; or combinations thereof.

[0076] In various embodiments of any of the aspects of the invention, a report displaying a determined ploidy status of a chromosome in a gestating fetus generated using the method. In some embodiments, a kit is disclosed for determining a ploidy status of a target chromosome in a gestating fetus designed to be used with any of the methods of the invention, the kit including a plurality of inner forward primers and optionally the plurality of inner reverse primers, where each of the primers is designed to hybridize to the region of DNA immediately upstream and/or downstream from one of the polymorphic sites on the target chromosome, and optionally additional chromosomes, where the region of hybridization is separated from the polymorphic site by a small number of bases, where the small number is selected from the group consisting of 1, 2, 3, 4, 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, 26 to 30, 31 to 60, and combinations thereof.

[0077] In one aspect, the invention features methods for establishing whether an alleged father is the biological father of a fetus that is gestating in a pregnant mother. In some embodiments the method involves, (i) simultaneously amplifying a plurality of polymorphic loci that includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different polymorphic loci on genetic material from the alleged father to produce a first set of amplified products; (ii) simultaneously amplifying the corresponding plurality of polymorphic loci on a mixed sample of DNA originating from a blood sample from the pregnant mother to produce a second set of amplified products; wherein the mixed sample of DNA includes fetal DNA and maternal DNA; (iii) determining on a computer the probability that the alleged father is the biological father of the fetus using genotypic measurements based on the first and second sets of amplified products; and (iv) establishing whether the alleged father is the biological father of the fetus using the determined probability that the alleged father is the biological father of the fetus. In various embodiments, the method further includes simultaneously amplifying the corresponding plurality of polymorphic loci on genetic material from the mother to produce a third set of amplified products; wherein the probability that the alleged father is the biological father of the fetus is determined using genotypic measurements based on the first, second, and third sets of amplified products.

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[0078] In one aspect, the invention provides methods of estimating relative likelihoods that each embryo from a set of embryos will develop as desired. In some embodiments, the method involves contacting a sample from each embryo with a library of primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture for each embryo, wherein the samples are each derived from one or more cells from an embryo. In some embodiments, each reaction mixture is subjected to primer extension reaction conditions to produce amplified products. In some embodiments, the method includes determining on a computer one or more characteristics of at least one cell from each embryo based on the amplified products; and estimating on a computer the relative likelihoods that each embryo will develop as desired, based on the one or more characteristics of the at least one cell for each embryo.

[0079] In one aspect, the invention features methods of measuring the amount of two or more target loci in a nucleic acid sample. In some embodiments, the method involves (i) using PCR to amplify a nucleic acid sample that includes a first standard locus, a second standard locus, a first target locus, and a second target locus to form amplified products; wherein the first standard locus and the first target locus have the same number of nucleotides but have a sequence that differs at one or more nucleotides; and wherein the second standard locus and the second target locus have the same number of nucleotides but have a sequence that differs at one or more nucleotides; (ii) sequencing the amplified products to determine a standard ratio that compares the relative amount of the amplified first standard locus compared to the amplified second standard locus; wherein the standard ratio indicates the difference in PCR efficiency for the amplification of the first standard locus and the second standard locus; (iii) determining a target ratio that compares the relative amount of the amplified first target locus compared to the amplified second target locus; and (iv) adjusting the target ratio from step (iii) based on the standard ratio from step (ii) to determine the relative amount of the first target locus and the second target locus in the sample. In various embodiments, the method involves determining the absolute amount of the first target locus and the second target locus in the sample. In various embodiments, the method further includes determining the presence or absence of a target locus (*e.g*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci) in the sample. In various embodiments, the method

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involves using any of the primer libraries of the invention. In various embodiments, the method involves simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci.

[0080] In one aspect, the invention features methods of quantitatively measuring a plurality of genetic targets in a sample for analysis. In some embodiments, the method includes (i) mixing genetic material derived from the sample for analysis with a plurality of target specific amplification reagents, and a plurality of standard sequences corresponding to the target specific amplification reagent targets; (ii) amplifying target regions of the genetic material and the standard sequences to produce target amplicons and standard sequence amplicons; and (iii) measuring the quantity of target amplicons and standard sequence amplicons produced. In some embodiments, the genetic material is present in a genetic library. In some embodiments, the genetic targets are polymorphic loci (such as SNPs). In some embodiments, the measuring of quantity is achieved by counting sequences. In some embodiments, the method further includes determining the estimated copy number of at least one chromosome in a sample from which the genetic library was derived, wherein the determination involves comparing the number of sequence reads of a target amplicon with the number of sequence reads of a standard amplicon. In some embodiments, the standard sequences and the genetic library include universal priming sites cable of being primed by the same primer. In some embodiments, the mixing step includes at least 10; 100, 500; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target specific amplification reagents and at least 10; 100, 500; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 standard sequences. In various embodiments, the method involves using any of the primer libraries of the invention. In various embodiments, the method involves simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target regions. In some embodiments, the relative amounts of each of the standard sequences are known. In some embodiments, the relative amounts of each of the sequences is has been calibrated with respect to a reference genome. In some embodiments, the sample for analysis includes a mixture of fetal and maternal genomes. In some embodiments, the sample for analysis is derived from the blood of a pregnant woman or

derived from blood plasma. In some embodiments, the reference genome has at least one aneuploidy, such as an aneuploidy at chromosome 13, 18, 21, X, or Y. In some embodiments, the reference genome is diploid.

[0081] In one aspect, the invention features a mixture that includes a plurality of genetic standard sequences, wherein the relative amount of each genetic standard sequence in the mixture has been determined by calibration to a reference genome. In various embodiments, the mixture includes at least 10; 100, 500; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 genetic standard sequences. In various embodiments, the genetic standard sequences include a first universal priming site, a second universal priming site, a first target specific priming site, a second target specific priming site, and a marker sequence located between the first and second target specific priming sites, wherein the first target specific site and the second target specific priming site are located between the first and second universal priming sites. In various embodiments, the calibration involves using any of the primer libraries of the invention. In various embodiments, the calibration involves simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target regions. In some embodiments, the reference genome has at least one aneuploidy, such as an aneuploidy at chromosome 13, 18, 21, X, or Y. In some embodiments, the reference genome is diploid.

[0082] In one aspect, the invention features methods of producing a set of calibrated genetic standard sequences. In some embodiments, the method includes (i) forming an amplification reaction mixture that includes a genetic library prepared from a reference genome, a plurality of target-specific amplification primer reagent sets, and a plurality of genetic standard sequences corresponding to the target specific amplification reagent sets, (ii) amplifying the genetic library and the genetic standard sequences to produce amplicons from the target sequences and amplicons from the genetic standard sequences, (iii) measuring the quantity of the amplicons from the target sequences and amplicons from the genetic standard sequences, and (iv) determining the relative amount of each of genetic standard sequences with respect to each other, whereby the plurality of genetic standard sequences is calibrated. In various embodiments, at least 10; 100, 500; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 genetic standard sequences are used. In various embodiments, the method involves using any of the primer

libraries of the invention. In various embodiments, the method involves simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different sequences. In some embodiments, the reference genome has at least one aneuploidy, such as an aneuploidy at chromosome 13, 18, 21, X, or Y. In some embodiments, the reference genome is diploid.

[0083] In one aspect, the invention provides a set of genetic standard sequences that have been calibrated according to any of the methods of the invention. In one aspect, the invention provides a set of genetic standard sequences that may be calibrated either before, during or after the method is performed.

[0084] In one aspect, the invention features methods of measuring the number of copies of a gene of interest having at least one allele that has a deletion. In some embodiments, the method includes (i) mixing genetic material derived from a sample for analysis with an amplification reagent specific for the gene of interest and not capable of significantly amplifying the deletion comprising allele of the gene of interest, a standard sequence corresponding to gene of interest, an amplification reagent specific for a reference sequence, and a standard sequence corresponding to the reference sequence; (ii) amplifying the gene sequence of interest, the standard sequence corresponding to the gene of interest, the reference sequence, and the standard sequence corresponding to the reference sequence to produce gene of interest amplicons, reference sequence amplicons, and standard sequence amplicons; and (iii) measuring the quantity of target amplicons and standard sequence amplicons produced. In some embodiments, the measuring of quantity is achieved by counting sequence reads. In some embodiments, the method further includes determining the estimated copy number of at least one chromosome in the sample from which the genetic library was derived, wherein the determination involves comparing the number of sequences of target amplicons with the number of sequences of a standard amplicons. In some embodiments, the standard sequences and the genetic library include universal priming sites capable of being primed by the same primer. In some embodiments, the relative amounts of each of the sequences have been calibrated with respect to reference genome. In various embodiments, at least 10; 100, 500; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 genetic standard sequences are used. In various embodiments, the method involves using any of the primer libraries of the invention. In

various embodiments, the method involves simultaneously amplifying at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target regions. In some embodiments, the reference genome is diploid. In some embodiments, the sample for analysis is derived from blood.

[0085] In some embodiments of any of the aspects of the invention, preferentially enriching the DNA in the sample (*e.g.*, the first sample) at the target loci (*e.g.*, the plurality of polymorphic loci) includes obtaining a plurality of pre-circularized probes where each probe targets one of the loci (*e.g.*, polymorphic loci), where the 3' and 5' end of the probes are preferably designed to hybridize to a region of DNA that is separated from the polymorphic site of the locus by a small number of bases, where the small number is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 to 25, 26 to 30, 31 to 60, or a combination thereof, hybridizing the pre-circularized probes to DNA from the sample (*e.g.*, the first sample), filling the gap between the hybridized probe ends using DNA polymerase, circularizing the pre-circularized probe, and amplifying the circularized probe.

[0086] In some embodiments of any of the aspects of the invention, the preferentially enriching the DNA at the target loci (*e.g.*, the plurality of polymorphic loci) includes obtaining a plurality of ligation-mediated PCR probes where each PCR probe targets one of the target loci (*e.g.*, the polymorphic loci), and where the upstream and downstream PCR probes are designed to hybridize to a region of DNA on one strand of DNA that is preferably separated from the polymorphic site of the locus by a small number of bases, where the small number is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 to 25, 26 to 30, 31 to 60, or a combination thereof, hybridizing the ligation-mediated PCR probes to the DNA from the sample (*e.g.*, the first sample), filling the gap between the ligation-mediated PCR probe ends using DNA polymerase, ligating the ligation-mediated PCR probes, and amplifying the ligated ligation-mediated PCR probes.

[0087] In some embodiments of various aspects of the invention, preferentially enriching the DNA at the target loci (*e.g.*, plurality of polymorphic loci) includes obtaining a plurality of hybrid capture probes that target the loci (*e.g.*, the polymorphic loci), hybridizing the hybrid capture probes to the DNA in the sample (*e.g.*, the first sample) and physically removing some or all of the unhybridized DNA from the sample (*e.g.*, the first sample) of DNA.

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[0088] In some embodiments of any of the aspects of the invention, the hybrid capture probes are designed to hybridize to a region that is flanking but not overlapping the polymorphic site. In some embodiments, the hybrid capture probes are designed to hybridize to a region that is flanking but not overlapping the polymorphic site, and where the length of the flanking capture probe may be selected from the group consisting of less than about 120 bases, less than about 110 bases, less than about 100 bases, less than about 90 bases, less than about 80 bases, less than about 70 bases, less than about 60 bases, less than about 50 bases, less than about 40 bases, less than about 30 bases, and less than about 25 bases. In some embodiments, the hybrid capture probes are designed to hybridize to a region that overlaps the polymorphic site, and where the plurality of hybrid capture probes comprise at least two hybrid capture probes for each polymorphic loci, and where each hybrid capture probe is designed to be complementary to a different allele at that polymorphic locus.

[0089] In some embodiments of any of the aspects of the invention, preferentially enriching the DNA a plurality of polymorphic loci includes obtaining a plurality of inner forward primers where each primer targets one of the polymorphic loci, and where the 3' end of the inner forward primers are designed to hybridize to a region of DNA upstream from the polymorphic site, and separated from the polymorphic site by a small number of bases, where the small number is selected from the group consisting of 1, 2, 3, 4, 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, 26 to 30, or 31 to 60 base pairs, optionally obtaining a plurality of inner reverse primers where each primer targets one of the polymorphic loci, and where the 3' end of the inner reverse primers are designed to hybridize to a region of DNA upstream from the polymorphic site, and separated from the polymorphic site by a small number of bases, where the small number is selected from the group consisting of 1, 2, 3, 4, 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, 26 to 30, or 31 to 60 base pairs, hybridizing the inner primers to the DNA, and amplifying the DNA using the polymerase chain reaction to form amplicons.

[0090] In some embodiments of any of the aspects of the invention, the method also includes obtaining a plurality of outer forward primers where each primer targets one of the target (*e.g.*, polymorphic loci), and where the outer forward primers are designed to hybridize to the region of DNA upstream from the inner forward primer, optionally obtaining a plurality of outer reverse primers where each primer targets one of the target loci (*e.g.*, polymorphic loci), and where the outer reverse primers are designed to hybridize to the region of DNA immediately

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downstream from the inner reverse primer, hybridizing the first primers to the DNA, and amplifying the DNA using the polymerase chain reaction.

[0091] In some embodiments of any of the aspects of the invention, the method also includes obtaining a plurality of outer reverse primers where each primer targets one of the polymorphic loci, and where the outer reverse primers are designed to hybridize to the region of DNA immediately downstream from the inner reverse primer, optionally obtaining a plurality of outer forward primers where each primer targets one of the target loci (*e.g.*, the polymorphic loci), and where the outer forward primers are designed to hybridize to the region of DNA upstream from the inner forward primer, hybridizing the first primers to the DNA, and amplifying the DNA using the polymerase chain reaction.

[0092] In some embodiments of any of the aspects of the invention, preparing the sample (*e.g.*, the first sample) further includes appending universal adapters to the DNA in the sample (*e.g.*, the first sample) and amplifying the DNA in the sample (*e.g.*, the first sample) using the polymerase chain reaction. In some embodiments, at least a fraction of the amplicons that are amplified are less than 100 bp, less than 90 bp, less than 80 bp, less than 70 bp, less than 65 bp, less than 60 bp, less than 55 bp, less than 50 bp, or less than 45 bp, and where the fraction is 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 99%.

[0093] In some embodiments of any of the aspects of the invention, amplifying the DNA is done in one or a plurality of individual reaction volumes, and where each individual reaction volume contains more than 100 different forward and reverse primer pairs, more than 200 different forward and reverse primer pairs, more than 500 different forward and reverse primer pairs, more than 1,000 different forward and reverse primer pairs, more than 2,000 different forward and reverse primer pairs, more than 5,000 different forward and reverse primer pairs, more than 10,000 different forward and reverse primer pairs, more than 20,000 different forward and reverse primer pairs, more than 50,000 different forward and reverse primer pairs, or more than 100,000 different forward and reverse primer pairs. In various embodiments of any of the aspects of the invention, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primer pairs are used.

[0094] In some embodiments of any of the aspects of the invention, preparing the sample (*e.g.*, the first sample) further comprises dividing the sample (*e.g.*, the first sample) into a plurality of portions, and where the DNA in each portion is preferentially enriched at a subset of the target

loci (*e.g.*, plurality of polymorphic loci). In some embodiments, the inner primers are selected by identifying primer pairs likely to form undesired primer duplexes and removing from the plurality of primers at least one of the pair of primers identified as being likely to form undesired primer duplexes. In some embodiments, the inner primers contain a region that is designed to hybridize either upstream or downstream of the targeted locus (*e.g.*, the polymorphic locus), and optionally contain a universal priming sequence designed to allow PCR amplification. In some embodiments, at least some of the primers additionally contain a random region that differs for each individual primer molecule. In some embodiments, at least some of the primers additionally contain a molecular barcode.

[0095] In some embodiments of any of the aspects of the invention, preferential enrichment results in average degree of allelic bias between the prepared sample and the sample (*e.g.*, the first sample) of a factor selected from the group consisting of no more than a factor of 2, no more than a factor of 1.5, no more than a factor of 1.2, no more than a factor of 1.1, no more than a factor of 1.05, no more than a factor of 1.02, no more than a factor of 1.01, no more than a factor of 1.005, no more than a factor of 1.002, no more than a factor of 1.001 and no more than a factor of 1.0001. In some embodiments, the plurality of polymorphic loci are SNPs. In some embodiments, measuring the DNA in the prepared sample is done by sequencing.

[0096] In some embodiments, the nucleic acids in the sample are non-specifically amplified prior to amplification of the target loci (such as specific amplification of the target loci with a primer library of the invention). In some embodiments, the non-specific amplification includes whole genome application (WGA), such as ligation-mediated PCR (LM-PCR), degenerate oligonucleotide primer PCR (DOP-PCR), or multiple displacement amplification (MDA). In some embodiments, the non-specific amplification includes universal PCR, such as adaptor-mediated universal PCR.

[0097] In some embodiments of any of the aspects of the invention, the target loci are present on the same nucleic acid of interest (*e.g*, the same chromosome or the same region of a chromosome). In some embodiments, at least some of the target loci are present on different nucleic acids of interest (*e.g*, different chromosomes). In some embodiments, the nucleic acid sample includes fragmented or digested nucleic acids. In some embodiments, the nucleic acid sample includes DNA, such as genomic DNA, cDNA, cell-free DNA (cfDNA), cell-free mitochondrial DNA (cf mDNA), cell-free DNA that originated from nuclear DNA (cf nDNA), cellular DNA, or mitochondrial DNA. In some embodiments, nucleic acid sample includes

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RNA, such as cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA. In some embodiments, the nucleic acid sample includes DNA from a single cell, 2 cells, 3 cells, 4 cells, 5 cells, 6 cells, 7 cells, 8 cells, 9 cell, 10 cells, or more than 10 cells. In some embodiments, the nucleic acid sample is a blood or plasma sample that is substantially free of cells. In some embodiments, the nucleic acid sample includes or is derived from blood, plasma, saliva, semen, sperm, cell culture supernatant, mucus secretion, dental plaque, gastrointestinal tract tissue, stool, urine, hair, bone, body fluids, , tears, tissue, skin, fingernails, blastomeres, embryos, amniotic fluid, chorionic villus samples, bile, lymph, cervical mucus, or a forensic sample. In some embodiments, the target loci are segments of human nucleic acids. In some embodiments, the target loci are segments of human nucleic acids found in the human genome. In some embodiments, the target loci comprise or consist of single nucleotide polymorphisms (SNPs). In some embodiments, the primers are DNA molecules.

[0098] In some embodiments of any of the aspects of the invention, the DNA in the sample (*e.g.*, the first sample) originates from maternal plasma. In some embodiments, preparing the sample (*e.g.*, the first sample) further comprises amplifying the DNA. In some embodiments, preparing the sample (*e.g.*, the first sample) further comprises preferentially enriching the DNA in the sample (*e.g.*, the first sample) at the target loci (*e.g.*, a plurality of polymorphic loci).

[0099] In various embodiments, the primer extension reaction or the polymerase chain reaction includes the addition of one or more nucleotides by a polymerase. In some embodiments, greater than or equal to 5, 10, 20, 30, 40, 50, or 60 cycles of PCR are performed. In some embodiments, the amplification of loci is performed using a polymerase (*e.g.*, a DNA polymerase, RNA polymerase, or reverse transcriptase) with low 5′→ 3′ exonuclease and/or low strand displacement activity. In some embodiments, a DNA polymerase is used produce DNA amplicons using DNA as a template. In some embodiments, a RNA polymerase is used produce RNA amplicons using DNA as a template. In some embodiments, a reverse transcriptase is used produce cDNA amplicons using RNA as a template.

[0100] In various embodiments, the primer extension reaction or the polymerase chain reaction does not include ligation-mediated PCR. In various embodiments, the primer extension reaction or the polymerase chain reaction does not include the joining of two primers by a ligase. In various embodiments, the primers do not include Linked Inverted Probes (LIPs),

which can also be called pre-circularized probes, pre-circularizing probes, circularizing probes, Padlock Probes, or Molecular Inversion Probes (MIPs). In some embodiments, the primers are not loopable primers. In some embodiments, the primers do not form a loop structure, for example, the primers do not comprise a 3' target specific portion, a stem (comprising a first loop forming region and a second loop forming region), and a loop portion. In various embodiments, the primer library, composition, kit, or method does not include an array (such as a microarray) or do no use an array (such as a microarray). In some embodiments, multiplex PCR and/or sequencing is performed without use of an array (such as a microarray). In some embodiments, the primer library, composition, kit, or method comprises a microarray. In some embodiments, the primers or the target loci do not comprise an STR allele (for example, the target loci may be non-polymorphic loci or the loci may comprise a polymorphism other than an STR allele). In some embodiments, some or all of the target loci comprise an STR allele.

[0101] It is understood that all of the aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments. It is understood that aspects and embodiments of the invention described herein include combinations of any two or more of the aspects or embodiments of the invention.

Definitions

[0102] *Single Nucleotide Polymorphism (SNP)* refers to a single nucleotide that may differ between the genomes of two members of the same species. The usage of the term should not imply any limit on the frequency with which each variant occurs.

[0103] *Sequence* refers to a DNA sequence or a genetic sequence. It may refer to the primary, physical structure of the DNA molecule or strand in an individual. It may refer to the sequence of nucleotides found in that DNA molecule, or the complementary strand to the DNA molecule. It may refer to the information contained in the DNA molecule as its representation *in silico*.

[0104] *Locus* refers to a particular region of interest on the DNA (or corresponding RNA) of an individual, which may refer to a SNP, the site of a possible insertion or deletion, or the site of some other relevant genetic variation. Disease-linked SNPs may also refer to disease-linked loci.

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[0105] *Polymorphic Allele,* also "Polymorphic Locus," refers to an allele or locus where the genotype varies between individuals within a given species. Some examples of polymorphic alleles include single nucleotide polymorphisms, short tandem repeats, deletions, duplications, and inversions.

[0106] *Polymorphic Site* refers to the specific nucleotides found in a polymorphic region that vary between individuals.

[0107] *Allele* refers to the alternative form or version of a gene that occupies a particular locus. *Genetic Data* also "*Genotypic Data"* refers to the data describing aspects of the genome of one or more individuals. It may refer to one or a set of loci, partial or entire sequences, partial or entire chromosomes, or the entire genome. It may refer to the identity of one or a plurality of nucleotides; it may refer to a set of sequential nucleotides, or nucleotides from different locations in the genome, or a combination thereof. Genotypic data is typically *in silico*, however, it is also possible to consider physical nucleotides in a sequence as chemically encoded genetic data. Genotypic Data may be said to be "on," "of," "at," "from" or "on" the individual(s). Genotypic Data may refer to output measurements from a genotyping platform where those measurements are made on genetic material.

[0108] *Genetic Material* also "*Genetic Sample"* refers to physical matter, such as tissue or blood, from one or more individuals comprising DNA or RNA

[0109] *Noisy Genetic Data* refers to genetic data with any of the following: allele dropouts, uncertain base pair measurements, incorrect base pair measurements, missing base pair measurements, uncertain measurements of insertions or deletions, uncertain measurements of chromosome segment copy numbers, spurious signals, missing measurements, other errors, or combinations thereof.

[0110] *Confidence* refers to the statistical likelihood that the called SNP, allele, set of alleles, ploidy call, or determined number of chromosome segment copies correctly represents the real genetic state of the individual.

[0111] *Ploidy Calling*, also "Chromosome Copy Number Calling," or "Copy Number Calling" (CNC), may refer to the act of determining the quantity and/or chromosomal identity of one or more chromosomes present in a cell.

[0112] *Aneuploidy* refers to the state where the wrong number of chromosomes (*e.g.,* the wrong number of full chromosomes or the wrong number of chromosome segments, such as the presence of deletions or duplications of a chromosome segment) is present in a cell. In the

case of a somatic human cell it may refer to the case where a cell does not contain 22 pairs of autosomal chromosomes and one pair of sex chromosomes. In the case of a human gamete, it may refer to the case where a cell does not contain one of each of the 23 chromosomes. In the case of a single chromosome type, it may refer to the case where more or less than two homologous but non-identical chromosome copies are present, or where there are two chromosome copies present that originate from the same parent. In some embodiments, the deletion of a chromosome segment is a microdeletion.

[0113] *Ploidy State* refers to the quantity and/or chromosomal identity of one or more chromosomes types in a cell.

[0114] *Chromosome* may refer to a single chromosome copy, meaning a single molecule of DNA of which there are 46 in a normal somatic cell; an example is 'the maternally derived chromosome 18'. Chromosome may also refer to a chromosome type, of which there are 23 in a normal human somatic cell; an example is 'chromosome 18'.

[0115] *Chromosomal Identity* may refer to the referent chromosome number, *i.e.* the chromosome type. Normal humans have 22 types of numbered autosomal chromosome types, and two types of sex chromosomes. It may also refer to the parental origin of the chromosome. It may also refer to a specific chromosome inherited from the parent. It may also refer to other identifying features of a chromosome.

[0116] *The State of the Genetic Material* or simply "Genetic State" may refer to the identity of a set of SNPs on the DNA, to the phased haplotypes of the genetic material, and to the sequence of the DNA, including insertions, deletions, repeats and mutations. It may also refer to the ploidy state of one or more chromosomes, chromosomal segments, or set of chromosomal segments.

[0117] *Allelic Data* refers to a set of genotypic data concerning a set of one or more alleles. It may refer to the phased, haplotypic data. It may refer to SNP identities, and it may refer to the sequence data of the DNA, including insertions, deletions, repeats and mutations. It may include the parental origin of each allele.

[0118] *Allelic State* refers to the actual state of the genes in a set of one or more alleles. It may refer to the actual state of the genes described by the allelic data.

[0119] *Allelic Ratio* or allele ratio, refers to the ratio between the amount of each allele at a locus that is present in a sample or in an individual. When the sample was measured by sequencing, the allelic ratio may refer to the ratio of sequence reads that map to each allele at

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the locus. When the sample was measured by an intensity based measurement method, the allele ratio may refer to the ratio of the amounts of each allele present at that locus as estimated by the measurement method.

[0120] *Allele Count* refers to the number of sequences that map to a particular locus, and if that locus is polymorphic, it refers to the number of sequences that map to each of the alleles. If each allele is counted in a binary fashion, then the allele count will be whole number. If the alleles are counted probabilistically, then the allele count can be a fractional number.

[0121] *Allele Count Probability* refers to the number of sequences that are likely to map to a particular locus or a set of alleles at a polymorphic locus, combined with the probability of the mapping. Note that allele counts are equivalent to allele count probabilities where the probability of the mapping for each counted sequence is binary (zero or one). In some embodiments, the allele count probabilities may be binary. In some embodiments, the allele count probabilities may be set to be equal to the DNA measurements.

[0122] *Allelic Distribution,* or 'allele count distribution' refers to the relative amount of each allele that is present for each locus in a set of loci. An allelic distribution can refer to an individual, to a sample, or to a set of measurements made on a sample. In the context of sequencing, the allelic distribution refers to the number or probable number of reads that map to a particular allele for each allele in a set of polymorphic loci. The allele measurements may be treated probabilistically, that is, the likelihood that a given allele is present for a give sequence read is a fraction between 0 and 1, or they may be treated in a binary fashion, that is, any given read is considered to be exactly zero or one copies of a particular allele.

[0123] *Allelic Distribution Pattern* refers to a set of different allele distributions for different parental contexts. Certain allelic distribution patterns may be indicative of certain ploidy states.

[0124] *Allelic Bias* refers to the degree to which the measured ratio of alleles at a heterozygous locus is different to the ratio that was present in the original sample, such as a sample of DNA. The degree of allelic bias at a particular locus is equal to the observed allelic ratio at that locus, as measured, divided by the ratio of alleles in the original DNA or RNA sample at that locus. Allelic bias may be defined to be greater than one, such that if the calculation of the degree of allelic bias returns a value, x, that is less than 1, then the degree of allelic bias may be restated as 1/x. Allelic bias maybe due to amplification bias, purification bias, or some other phenomenon that affects different alleles differently.

[0125] *Allelic imbalance for aneuploidy determinations*, such as *CNV determinations*, refers to the difference between the frequencies of the alleles for a locus. It is an estimate of the difference in the copy of numbers of the homologs. Allelic imbalance can arise from the complete loss of an allele or from an increase in copy number of one allele relative to the other. Allelic imbalances can be detected by measuring the proportion of one allele relative to the other in fluids or cells from individuals that are constitutionally heterozygous at a given locus. (Mei et al, Genome Res, 10:1126-37 (2000)). For dimorphic SNPs that have alleles arbitrarily designated 'A' and 'B', the allele ratio of the A allele is $n_A/(n_A + n_B)$, where n_A and n_B are the number of sequencing reads for alleles A and B, respectively. Allelic imbalance is the difference between the allele ratios of A and B for loci that are heterozygous in the germline. This definition is analogous to that for SNVs, where the proportion of abnormal DNA is typically measured using mutant allele frequency, or $n_m/(n_m + n_r)$, where n_m and n_r are the number of sequencing reads for the mutant allele and the reference allele, respectively. Accordingly, the proportion of abnormal DNA for a CNV can be measured by the average allelic imbalance (AAI), defined as $|(H1 - H2)|/(H1 + H2)$, where Hi is the average number of copies of homolog i in the sample and $Hi/(H1 + H2)$ is the fractional abundance, or homolog ratio, of homolog i. The maximum homolog ratio is the homolog ratio of the more abundant homolog.

[0126] *Primer,* also "PCR probe" refers to a single DNA molecule (a DNA oligomer) or a collection of DNA molecules (DNA oligomers) where the DNA molecules are identical, or nearly so, and where the primer contains a region that is designed to hybridize to a targeted locus (*e.g,* a targeted polymorphic locus or a nonpolymorphic locus), and may contain a priming sequence designed to allow PCR amplification. A primer may also contain a molecular barcode. A primer may contain a random region that differs for each individual molecule. The terms "test primer" and "candidate primer" are not meant to be limiting and may refer to any of the primers disclosed herein.

[0127] *Library of primers* refers to a population of two or more primers. In various embodiments, the library includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers. In various embodiments, the library includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primer pairs,

wherein each pair of primers includes a forward test primer and a reverse test primer where each pair of test primers hybridize to a target locus. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different individual primers that each hybridize to a different target locus, wherein the individual primers are not part of primer pairs. In some embodiments, the library has both (i) primer pairs and (ii) individual primers (such as universal primers) that are not part of primer pairs.

[0128] *Different primers* refers to non-identical primers.

[0129] *Different pools* refers to non-identical pools.

[0130] *Different target loci* refers to non-identical target loci.

[0131] *Different amplicons* refers to non-identical amplicons.

[0132] *Hybrid Capture Probe* refers to any nucleic acid sequence, possibly modified, that is generated by various methods such as PCR or direct synthesis and intended to be complementary to one strand of a specific target DNA sequence in a sample. The exogenous hybrid capture probes may be added to a prepared sample and hybridized through a denature-reannealing process to form duplexes of exogenous-endogenous fragments. These duplexes may then be physically separated from the sample by various means.

[0133] *Sequence Read* refers to data representing a sequence of nucleotide bases that were measured using a clonal sequencing method. Clonal sequencing may produce sequence data representing single, or clones, or clusters of one original DNA molecule. A sequence read may also have associated quality score at each base position of the sequence indicating the probability that nucleotide has been called correctly.

[0134] *Mapping a sequence read* is the process of determining a sequence read's location of origin in the genome sequence of a particular organism. The location of origin of sequence reads is based on similarity of nucleotide sequence of the read and the genome sequence.

[0135] *Matched Copy Error*, also "Matching Chromosome Aneuploidy" (MCA), refers to a state of aneuploidy where one cell contains two identical or nearly identical chromosomes. This type of aneuploidy may arise during the formation of the gametes in meiosis, and may be referred to as a meiotic non-disjunction error. This type of error may arise in mitosis. Matching trisomy may refer to the case where three copies of a given chromosome are present in an individual and two of the copies are identical.

[0136] *Unmatched Copy Error*, also "Unique Chromosome Aneuploidy" (UCA), refers to a state of aneuploidy where one cell contains two chromosomes that are from the same parent, and that may be homologous but not identical. This type of aneuploidy may arise during meiosis, and may be referred to as a meiotic error. Unmatching trisomy may refer to the case where three copies of a given chromosome are present in an individual and two of the copies are from the same parent, and are homologous, but are not identical. Note that unmatching trisomy may refer to the case where two homologous chromosomes from one parent are present, and where some segments of the chromosomes are identical while other segments are merely homologous.

[0137] *Homologous Chromosomes* refers to chromosome copies that contain the same set of genes that normally pair up during meiosis.

[0138] *Identical Chromosomes* refers to chromosome copies that contain the same set of genes, and for each gene they have the same set of alleles that are identical, or nearly identical.

[0139] *Allele Drop Out (ADO)* refers to the situation where at least one of the base pairs in a set of base pairs from homologous chromosomes at a given allele is not detected.

[0140] *Locus Drop Out (LDO)* refers to the situation where both base pairs in a set of base pairs from homologous chromosomes at a given allele are not detected.

[0141] *Homozygous* refers to having similar alleles as corresponding chromosomal loci.

[0142] *Heterozygous* refers to having dissimilar alleles as corresponding chromosomal loci.

[0143] *Heterozygosity Rate* refers to the rate of individuals in the population having heterozygous alleles at a given locus. The heterozygosity rate may also refer to the expected or measured ratio of alleles, at a given locus in an individual, or a sample of DNA.

[0144] *Highly Informative Single Nucleotide Polymorphism (HISNP)* refers to a SNP where the fetus has an allele that is not present in the mother's genotype.

[0145] *Chromosomal Region* refers to a segment of a chromosome, or a full chromosome.

[0146] *Segment of a Chromosome* refers to a section of a chromosome that can range in size from one base pair to the entire chromosome.

[0147] *Chromosome* refers to either a full chromosome, or a segment or section of a chromosome.

[0148] *Copies* refers to the number of copies of a chromosome segment. It may refer to identical copies, or to non-identical, homologous copies of a chromosome segment wherein the different copies of the chromosome segment contain a substantially similar set of loci, and

where one or more of the alleles are different. Note that in some cases of aneuploidy, such as the M2 copy error, it is possible to have some copies of the given chromosome segment that are identical as well as some copies of the same chromosome segment that are not identical.

[0149] *Haplotype* refers to a combination of alleles at multiple loci that are typically inherited together on the same chromosome. Haplotype may refer to as few as two loci or to an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. Haplotype can also refer to a set of single nucleotide polymorphisms (SNPs) on a single chromatid that are statistically associated.

[0150] *Haplotypic Data*, also "Phased Data" or "Ordered Genetic Data," refers to data from a single chromosome in a diploid or polyploid genome, *i.e.*, either the segregated maternal or paternal copy of a chromosome in a diploid genome.

[0151] *Phasing* refers to the act of determining the haplotypic genetic data of an individual given unordered, diploid (or polyploidy) genetic data. It may refer to the act of determining which of two genes at an allele, for a set of alleles found on one chromosome, are associated with each of the two homologous chromosomes in an individual.

[0152] *Phased Data* refers to genetic data where one or more haplotypes have been determined.

[0016] *Hypothesis* refers to a possible ploidy state at a given set of chromosomes, or a set of possible allelic states at a given set of loci. The set of possibilities may comprise one or more elements.

[0153] *Copy Number Hypothesis*, also "Ploidy State Hypothesis," refers to a hypothesis concerning the number of copies of a chromosome in an individual. It may also refer to a hypothesis concerning the identity of each of the chromosomes, including the parent of origin of each chromosome, and which of the parent's two chromosomes are present in the individual. It may also refer to a hypothesis concerning which chromosomes, or chromosome segments, if any, from a related individual correspond genetically to a given chromosome from an individual.

[0154] *Target Individual* refers to the individual whose genetic state is being determined. In some embodiments, only a limited amount of DNA is available from the target individual. In some embodiments, the target individual is a fetus. In some embodiments, there may be more than one target individual. In some embodiments, each fetus that originated from a pair of parents may be considered to be target individuals. In some embodiments, the genetic data that

is being determined is one or a set of allele calls. In some embodiments, the genetic data that is being determined is a ploidy call.

[0155] *Related Individual* refers to any individual who is genetically related to, and thus shares haplotype blocks with, the target individual. In one context, the related individual may be a genetic parent of the target individual, or any genetic material derived from a parent, such as a sperm, a polar body, an embryo, a fetus, or a child. It may also refer to a sibling, parent or a grandparent.

[0156] *Sibling* refers to any individual whose genetic parents are the same as the individual in question. In some embodiments, it may refer to a born child, an embryo, or a fetus, or one or more cells originating from a born child, an embryo, or a fetus. A sibling may also refer to a haploid individual that originates from one of the parents, such as a sperm, a polar body, or any other set of haplotypic genetic matter. An individual may be considered to be a sibling of itself.

[0157] *Fetal* refers to "of the fetus," or "of the region of the placenta that is genetically similar to the fetus". In a pregnant woman, some portion of the placenta is genetically similar to the fetus, and the free floating fetal DNA found in maternal blood may have originated from the portion of the placenta with a genotype that matches the fetus. Note that the genetic information in half of the chromosomes in a fetus is inherited from the mother of the fetus. In some embodiments, the DNA from these maternally inherited chromosomes that came from a fetal cell is considered to be "of fetal origin," not "of maternal origin."

[0158] *DNA of Fetal Origin* refers to DNA that was originally part of a cell whose genotype was essentially equivalent to that of the fetus.

[0159] *DNA of Maternal Origin* refers to DNA that was originally part of a cell whose genotype was essentially equivalent to that of the mother.

[0160] *Child* may refer to an embryo, a blastomere, or a fetus. Note that in the presently disclosed embodiments, the concepts described apply equally well to individuals who are a born child, a fetus, an embryo or a set of cells therefrom. The use of the term child may simply be meant to connote that the individual referred to as the child is the genetic offspring of the parents.

[0161] *Parent* refers to the genetic mother or father of an individual. An individual typically has two parents, a mother and a father, though this may not necessarily be the case such as in genetic or chromosomal chimerism. A parent may be considered to be an individual.

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[0162] *Parental Context* refers to the genetic state of a given SNP, on each of the two relevant chromosomes for one or both of the two parents of the target.

[0163] *Develop As Desired*, also "Develop Normally," refers to a viable embryo implanting in a uterus and resulting in a pregnancy, and/or to a pregnancy continuing and resulting in a live birth, and/or to a born child being free of chromosomal abnormalities, and/or to a born child being free of other undesired genetic conditions such as disease-linked genes. The term "develop as desired" is meant to encompass anything that may be desired by parents or healthcare facilitators. In some cases, "develop as desired" may refer to an unviable or viable embryo that is useful for medical research or other purposes.

[0164] *Insertion into a Uterus* refers to the process of transferring an embryo into the uterine cavity in the context of *in vitro* fertilization.

[0165] *Maternal Plasma* refers to the plasma portion of the blood from a female who is pregnant.

[0166] *Clinical Decision* refers to any decision to take or not take an action that has an outcome that affects the health or survival of an individual. In the context of prenatal diagnosis, a clinical decision may refer to a decision to abort or not abort a fetus. A clinical decision may also refer to a decision to conduct further testing, to take actions to mitigate an undesirable phenotype, or to take actions to prepare for the birth of a child with abnormalities.

[0167] *Diagnostic Box* refers to one or a combination of machines designed to perform one or a plurality of aspects of the methods disclosed herein. In an embodiment, the diagnostic box may be placed at a point of patient care. In an embodiment, the diagnostic box may perform targeted amplification followed by sequencing. In an embodiment the diagnostic box may function alone or with the help of a technician.

[0168] *Informatics Based Method* refers to a method that relies heavily on statistics to make sense of a large amount of data. In the context of prenatal diagnosis, it refers to a method designed to determine the ploidy state at one or more chromosomes or the allelic state at one or more alleles by statistically inferring the most likely state, rather than by directly physically measuring the state, given a large amount of genetic data, for example from a molecular array or sequencing. In an embodiment of the present disclosure, the informatics based technique may be one disclosed in this patent. In an embodiment of the present disclosure it may be PARENTAL SUPPORT™.

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[0169] *Primary Genetic Data* refers to the analog intensity signals that are output by a genotyping platform. In the context of SNP arrays, primary genetic data refers to the intensity signals before any genotype calling has been done. In the context of sequencing, primary genetic data refers to the analog measurements, analogous to the chromatogram, that comes off the sequencer before the identity of any base pairs have been determined, and before the sequence has been mapped to the genome.

[0170] *Secondary Genetic Data* refers to processed genetic data that are output by a genotyping platform. In the context of a SNP array, the secondary genetic data refers to the allele calls made by software associated with the SNP array reader, wherein the software has made a call whether a given allele is present or not present in the sample. In the context of sequencing, the secondary genetic data refers to the base pair identities of the sequences have been determined, and possibly also where the sequences have been mapped to the genome.

[0171] *Non-Invasive Prenatal Diagnosis (NPD)*, or also "Non-Invasive Prenatal Screening" (NPS), refers to a method of determining the genetic state of a fetus that is gestating in a mother using genetic material found in the mother's blood, where the genetic material is obtained by drawing the mother's intravenous blood.

[0172] *Preferential Enrichment* of DNA that corresponds to a locus, or preferential enrichment of DNA at a locus, refers to any method that results in the percentage of molecules of DNA in a post-enrichment DNA mixture that correspond to the locus being higher than the percentage of molecules of DNA in the pre-enrichment DNA mixture that correspond to the locus. The method may involve selective amplification of DNA molecules that correspond to a locus. The method may involve removing DNA molecules that do not correspond to the locus. The method may involve a combination of methods. The degree of enrichment is defined as the percentage of molecules of DNA in the post-enrichment mixture that correspond to the locus divided by the percentage of molecules of DNA in the pre-enrichment mixture that correspond to the locus. Preferential enrichment may be carried out at a plurality of loci. In some embodiments of the present disclosure, the degree of enrichment is greater than 20. In some embodiments of the present disclosure, the degree of enrichment is greater than 200. In some embodiments of the present disclosure, the degree of enrichment is greater than 2,000. When preferential enrichment is carried out at a plurality of loci, the degree of enrichment may refer to the average degree of enrichment of all of the loci in the set of loci.

[0173] *Amplification* refers to a method that increases the number of copies of a molecule, such as a molecule of DNA.

[0174] *Selective Amplification* may refer to a method that increases the number of copies of a particular molecule of DNA, or molecules of DNA that correspond to a particular region of DNA. It may also refer to a method that increases the number of copies of a particular targeted molecule of DNA, or targeted region of DNA more than it increases non-targeted molecules or regions of DNA. Selective amplification may be a method of preferential enrichment.

[0175] *Universal Priming Sequence* refers to a DNA sequence that may be appended to a population of target DNA molecules, for example by ligation, PCR, or ligation mediated PCR. Once added to the population of target molecules, primers specific to the universal priming sequences can be used to amplify the target population using a single pair of amplification primers. Universal priming sequences are typically not related to the target sequences.

[0176] *Universal Adapters,* or 'ligation adaptors' or 'library tags' are DNA molecules containing a universal priming sequence that can be covalently linked to the 5-prime and 3-prime end of a population of target double stranded DNA molecules. The addition of the adapters provides universal priming sequences to the 5-prime and 3-prime end of the target population from which PCR amplification can take place, amplifying all molecules from the target population, using a single pair of amplification primers.

[0177] *Targeting* refers to a method used to selectively amplify or otherwise preferentially enrich those molecules of DNA that correspond to a set of loci, in a mixture of DNA.

[0178] *Joint Distribution Model* refers to a model that defines the probability of events defined in terms of multiple random variables, given a plurality of random variables defined on the same probability space, where the probabilities of the variable are linked. In some embodiments, the degenerate case where the probabilities of the variables are not linked may be used.

[0179] *Percent identity* in reference to nucleic acid sequences refers to the degree of sequence identity between nucleic acid sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

[0180] The presently disclosed embodiments will be further explained with reference to the attached drawings, wherein like structures are referred to by like numerals throughout the

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several views. The drawings shown are not necessarily to scale, with emphasis instead generally being placed upon illustrating the principles of the presently disclosed embodiments.

[0181] FIG. 1: Graphical representation of direct multiplexed mini-PCR method.

[0182] FIG. 2: Graphical representation of semi-nested mini-PCR method.

[0183] FIG. 3: Graphical representation of fully nested mini-PCR method.

[0184] FIG. 4: Graphical representation of hemi-nested mini-PCR method.

[0185] FIG. 5: Graphical representation of triply hemi-nested mini-PCR method.

[0186] FIG. 6: Graphical representation of one-sided nested mini-PCR method.

[0187] FIG. 7: Graphical representation of one-sided mini-PCR method.

[0188] FIG. 8: Graphical representation of reverse semi-nested mini-PCR method.

[0189] FIG. 9: Some possible workflows for semi-nested methods.

[0190] FIG. 10: Graphical representation of looped ligation adaptors.

[0191] FIG. 11: Graphical representation of internally tagged primers.

[0192] FIG. 12: An example of some primers with internal tags (SEQ ID NOs: 44,611-44,622, respectively in order of appearance).

[0193] FIG. 13: Graphical representation of a method using primers with a ligation adaptor binding region.

[0194] FIG. 14: Simulated ploidy call accuracies for counting method with two different analysis techniques.

[0195] FIG. 15: Ratio of two alleles for a plurality of SNPs in a cell line in Example 4.

[0196] FIG. 16: Ratio of two alleles for a plurality of SNPs in a cell line in Example 4 sorted by chromosome.

[0197] FIGs. 17A-17D: Ratio of two alleles for a plurality of SNPs in four pregnant women plasma samples, sorted by chromosome.

[0198] FIG. 18: Fraction of data that can be explained by binomial variance before and after data correction.

[0199] FIG. 19: Graph showing relative enrichment of fetal DNA in samples following a short library preparation protocol.

[0200] FIG. 20: Depth of read graph comparing direct PCR and semi-nested methods.

[0201] FIG. 21: Comparison of depth of read for direct PCR of three genomic samples.

[0202] FIG. 22: Comparison of depth of read for semi-nested mini-PCR of three samples.

[0203] FIG. 23: Comparison of depth of read for 1,200-plex and 9,600-plex reactions.

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[0204] FIG. 24: Read count ratios for six cells at three chromosomes.

[0205] FIGs. 25A-25C: Allele ratios for two three-cell reactions (FIGs. 25B and 25C) and a third reaction run on 1 ng of genomic DNA at three chromosomes (FIG. 25A).

[0206] FIGs. 26A and 26B: Allele ratios for two single-cell reactions (FIGs. 26A and 26B) at three chromosomes.

[0207] FIG. 27: Comparison of two primer libraries showing the number of loci with a particular minor allele frequency that are targeted by each primer library.

[0208] FIG. 28A: Graph of the electrophoresis of PCR products.

[0209] FIGs. 28B-28M are electropherograms of lanes 1-12, respectively, in FIG. 28A.

[0210] FIG. 29: Cartoon depiction of a method of the invention for the determination of a fetal aneuploidy (FIG. 29, step A). Maternal and paternal genotype data (from blood or buccal swabs) and crossover frequency data from the HapMap database are utilized to generate (FIG. 29, step B) multiple independent hypotheses for each potential fetal ploidy state *in silico* (FIG. 29, step C). Each of these hypotheses is expanded to include sub-hypotheses with take into consideration the different possible crossover points. The data model predicts what the sequencing data would look like (the expected allele distributions) given each hypothetical fetal genotype and at different fetal cfDNA fractions, and is compared to the actual sequencing data; the likelihood for each hypothesis is determined using Bayesian statistics. In this hypothetical example, the hypotheses with the highest likelihoods (euploidy) are determined (FIG. 29, step D). The individual likelihoods from FIG. 29, step C are summed for each copy number hypothesis family (monosomy, disomy, or triploidy). The hypothesis with the maximum likelihood is called as the ploidy state, reveals the fetal fraction, and represents the sample-specific calculated accuracy.

[0211] FIGs. 30, 30D-H: Typical graphical representations of euploidy (FIG. 30), monosomy (FIG. 30D), and trisomy (FIGs. 30E-30H). For all plots, the x-axis represents the linear position of the individual polymorphic loci along each chromosome (as indicated below the plots), and the y-axis represents the number of A allele reads as a fraction of the total (A+B) allele reads. Maternal and fetal genotypes, as well as the position on the y-axis around which the bands are centered, are indicated to the right of the plots. If desired to facilitate visualization, the plots may be color-coded according to maternal genotype, such that red (filled circles as shown in FIGS. 30, 30D-H) indicates a maternal genotype of AA, blue (filled squares as shown in FIGS. 30, 30D-H) indicates a maternal genotype of BB, and green (open

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triangles as shown in FIGS. 30, 30D-H) indicates a maternal genotype of AB. If desired, maternal allele contributions may be indicated in color in the "Fetal Genotype" column. Allele contributions are indicated as maternal|fetal, such that alleles for which the mother is AA and the fetus is AB are indicated as AA|AB. FIG. 30, 0% FF plot: Generated plots when two chromosomes are present and the fetal cfDNA fraction is 0%. This plot is from a non-pregnant woman, and thus represents the pattern when the genotype is entirely maternal. Allele clusters are thus centered around 1 (AA alleles), 0.5 (AB alleles), and 0 (BB alleles). FIG. 30, 12% FF plot**:** Generated plot when two chromosomes are present and the fetal fraction is 12%. The contribution of fetal alleles to the fraction of A allele reads shifts the position of some allele spots up or down along the y-axis, such that the bands are centered around 1 (AA|AA alleles), 0.94 (AA|AB alleles), 0.56 (AB|AA alleles), 0.50 (AB|AB alleles), 0.44 (AB|BB alleles), 0.06 (BB|AB alleles), and 0 (BB|BB alleles). FIG. 30, 26% FF Plot. Generated plot when two chromosomes are present and the fetal fraction is 26%. The pattern, including two filled circles and two filled square peripheral bands and a trio of central open triangle bands, is readily apparent. Bands are centered around 1 (AA|AA alleles), 0.87 (AA|AB alleles), 0.63 (AB|AA alleles), 0.50 (AB|AB alleles), 0.37 (AB|BB alleles), 0.13 (BB|AB alleles), and 0 (BB|BB alleles). FIG. 30D: Generated plot when one chromosome is present and the fetal fraction is 26%. The hallmark pattern of one external filled circles and one external filled square peripheral band as well as two central open triangle bands indicated maternally-inherited monosomy. Because the fetus only contributes a single allele (A or B) to the allele reads, the internal peripheral filled circles and filled square bands are not present, and the center trio of bands condenses into two bands. Bands that are centered around 1 (AA|A alleles), 0.57 (AB|A alleles), 0.43 (AB|B alleles), and 0 (BB|B alleles). FIG. 30E: Generated plot when three chromosomes are present and the fetal fraction is 27%. This pattern of two filled circles and filled square peripheral bands as well as two central open triangle bands indicates maternally-inherited meiotic trisomy. Bands are centered around 1 (AA|AAA alleles), 0.88 (AA|AAB alleles), 0.56 (AB|AAB alleles), 0.44 (AB|ABB alleles), 0.12 (BB|ABB alleles), and 0 (BB|BBB alleles). FIG. 30F: Generated plot when three chromosomes are present and the fetal fraction is 14%. This pattern of three filled circles and three filled square peripheral bands, as well as two central open triangle bands, indicates paternally-inherited meiotic trisomy. Bands are centered around 1 (AA|AAA alleles), 0.93 (AA|AAB alleles), 0.87 (AA|ABB alleles), 0.60 (AB|AAA alleles), 0.53 (AB|AAB alleles), 0.47 (AB|ABB alleles), 0.40 (AB|BBB alleles),

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0.13 (BB|AAB alleles), 0.07 (BB|ABB alleles), and 0 (BB|BBB alleles). FIG. 30G: Generated plot when three chromosomes are present and the fetal fraction is 35%. This pattern of two filled circles and two filled square peripheral bands and four central open triangle bands indicates maternally-inherited mitotic trisomy. Bands are centered around 1 (AA|AAA alleles), 0.85 (AA|AAB alleles), 0.72 (AB|AAA alleles), 0.57 (AB|AAB alleles), 0.43 (AB|ABB alleles), 0.28 (AB|BBB alleles), 0.15 (BB|ABB alleles), and 0 (BB|BBB alleles). FIG. 30H: Generated plot when three chromosomes are present and the fetal fraction is 25%. This pattern of two filled circles and two filled square peripheral bands as well as four central open trinagle bands indicates paternally-inherited mitotic trisomy. This pattern can be distinguished from that of maternally-inherited mitotic trisomy (as in FIG. 30G) by the position of the internal peripheral bands. Specifically, bands are centered around 1 (AA|AAA alleles), 0.78 (AA|ABB alleles), 0.67 (AB|AAA alleles), 0.56 (AB|AAB alleles), 0.44 (AB|ABB alleles), 0.33 (AB|BBB alleles), 0.22 (BB|AAB alleles), and 0 (BB|BBB alleles).

[0210] FIGs. 31A-31G: Graphical representations of (FIG. 31A) euploid, (FIG. 31B) T13, (FIG. 31C) T18, (FIG. 31D) T21, (FIG. 31E) 45,X, (FIG. 31F) 47,XXY, and (FIG. 31G) 47,XYY, test samples as indicated. Each chromosome is indicated at the top of the plot, fetal and maternal genotypes are indicated to the right of the plots, the x-axis represents the linear position of the SNPs along each chromosome, and the y-axis indicates the number of A allele reads as a fraction of the total reads. Note the altered cluster positioning based on fetal fraction, as described herein. Each spot represents a single SNP locus. Fetal and maternal genotypes are indicated to the right of the plot, and chromosome identities are indicated at the top of the plots.

[0211] FIG. 32: The combined at-birth prevalence of sex chromosome aneuploidies is greater than that of autosomal aneuploidies.

[0212] FIGs. 33A-33F: Illustrations of the calculation of an interaction score between primers in a primer library. FIG. 33A shows the first two bases (dinucleotide) of a primer that align to the other primer for calculation of ΔG. Iterate over the remainder of the primer that aligns with the other primer by sliding the bases being observing one base to the right. Continue until ΔG has been calculated for all pairs of bases that align (FIG. 33B). Shift the alignment of the two primers (FIGs. 33C and 33D). Determine ΔG for the new alignment (FIGs. 33E and 33F).

[0213] FIG. 34: Table of the percentage of reads that map to target loci for genomic DNA samples and for samples of a single cell from a cell line for both mother and child samples.

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[0214] FIG. 35: Overlay of depth of read for a genomic and a single cell sample for different SNPs.

[0215] FIG. 36: Table of the percentage of reads that map to target loci for blastoceol fluid and for a single blastocyst cell.

[0216] FIG. 37. Graph of reference counts (counts of one allele, such as the "A" allele) divided by total counts for that locus for a single blastocyst cell.

[0217] FIG. 38 is a graph showing the limit of detection for single nucleotide variants in a tumor biopsy using three different methods described in Example 23.

[0218] FIG. 39 is a graph showing the limit of detection for single nucleotide variants in a plasma sample using three different methods described in Example 23.

[0219] FIGs. 40A and 40B are graphs of the analysis of genomic DNA (FIG. 40A) or DNA from a single cell (FIG. 40B) using a library of approximately 28,000 primers designed to detect CNVs. The presence of two central bands instead of one central band indicates the presence of a CNV. The x-axis represents the linear position of the SNPs, and the y-axis indicates the fraction of A allele reads out of the total reads.

[0220] FIGs. 41A and 41B are graphs of the analysis of genomic DNA (FIG. 41A) or DNA from a single cell (FIG. 41B) using a library of approximately 3,000 primers designed to detect CNVs. The presence of two central bands instead of one central band indicates the presence of a CNV. The x-axis represents the linear position of the SNPs, and the y-axis indicates the fraction of A allele reads out of the total reads.

[0221] FIG. 42 is a graph illustrating the uniformity in depth of read (DOR) for these ~3,000 loci.

[0222] FIG. 43 is a table comparing error call metrics for genomic DNA and DNA from a single cell.

[0223] FIG. 44 is a graph of error rates for transition mutations and transversion mutations.

[0224] FIG. 45 is a table of data (such as percent mapped reads and error rate) from multiplex PCR with various buffers.

[0225] FIG. 46 is a graph illustrating the uniformity in DOR for multiplex PCR with buffers from FIG. 45.

[0226] FIG. 47 is a graph illustrating the normalized depth of read (DOR) for multiplex PCR with buffers from FIG. 45 with the DOR normalized to that of buffer 2xMM.

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[0227] While the above-identified drawings set forth presently disclosed embodiments, other embodiments are also contemplated, as noted in the discussion. This disclosure presents illustrative embodiments by way of representation and not limitation. Numerous other modifications and embodiments can be devised by those skilled in the art which fall within the scope and spirit of the principles of the presently disclosed embodiments.

DETAILED DESCRIPTION

[0228] The present invention is based in part on the surprising discovery that often only a relatively small number of primers in a library of primers are responsible for a substantial amount of the amplified primer dimers that form during multiplex PCR reactions. Methods have been developed to select the most undesirable primers for removal from a library of candidate primers. By reducing the amount of primer dimers to a negligible amount (~0.1% of the PCR products), these methods allow the resulting primer libraries to simultaneously amplify a large number of target loci in a single multiplex PCR reaction. Because the primers hybridize to the target loci and amplify them rather than hybridizing to other primers and forming amplified primer dimers, the number of different target loci that can be amplified is increased. It was also discovered that using lower primer concentrations and much longer annealing times than normal increases the likelihood that the primers hybridize to the target loci instead of hybridizing to each other and forming primer dimers (see, *e.g.*, U.S.S.N. 13/683,604, filed Nov. 21, 2012, which is hereby incorporated by reference in its entirety). The methods can also be used to amplify a large number of target loci even if the primers have a relatively large range of melting temperatures (in contrast to other methods that require primers to have very similar melting temperatures to successfully amplify multiple loci simultaneously due to the need for the primers to be functional under the same reaction conditions). Additionally, it was discovered that the annealing temperature can optionally be higher than the melting temperatures of the primers (in contrast to other methods that use an annealing temperature below the melting temperatures of the primers). A higher annealing temperature improves the specificity of the PCR amplification and reduces or prevents amplification of non-target loci.

[0229] During the PCR amplification and sequencing of 19,488 target loci in a genomic sample, 99.4-99.7% of the sequencing reads mapped to the genome, of those, 99.99% of the mapped to target loci. For plasma samples with 10 million sequencing reads, typically at least 19,350 of

the 19,488 target loci (99.3 %) were amplified and sequenced. This primer library has even been used to amplify the nucleic acids in a single cell (FIGs. 34-37).

[0230] During the PCR amplification and sequencing of ~28,000 target loci in a genomic sample, 99% of the sequencing reads mapped to target loci. This primer library has also been used to amplify nucleic acids in a single cell.

[0231] Being able to simultaneously amplify such a large number of target loci at once greatly decreases the amount of time and the amount of DNA required to analyze thousands of target loci. For example, DNA from a single cell is sufficient to simultaneously analyze thousands of target loci, which is important for applications in which the amount of DNA is low, such as genetic testing of a single cell from an embryo prior to *in vitro* fertilization or genetic testing of a forensic sample with little DNA. In addition, being able to analyze the target loci in one reaction volume (such as in one chamber, well, or vessel) rather than splitting the sample into multiple different reactions reduces variability that can occur between reactions. In addition, methods have been developed to use reference standards to correct for amplification bias that may occur between different target loci. For example, differences in amplification efficiency between target loci due to factors such as GC content may cause differing amounts of PCR products to be produced for target loci that are actually present in the same amount. The use of reference standards similar to the target loci allows the detection of such amplification bias so that it can be corrected for during the quantitation of the target loci.

[0232] During sequencing of PCR products, artifacts such as primer dimers are detected and thus inhibit the detection of target amplicons. Because of this limitation, microarrays with hybridization probes are often used for detection since microarrays are less sensitive to interference from primer dimers (for example, microarrays can be used as a target specific detection that uses probes to hybridize to target amplicons but does not have probes to hybridize to undesired primer dimers). The high level of multiplexing with minimal non-target amplicons that has now been achieved allows PCR followed by sequencing to be used as an alternative to microarrays. For example, the present multiplex PCR methods can be used with a non-target specific method of detection, such as sequencing that detects all amplified products (including both target amplicons and primer dimers, if any). The small amount of primer dimers that are produced allows detection of target amplicons by methods that detect all amplicons. Thus, in some embodiments, the method includes multiplex PCR followed by sequencing without use of

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an array. In other embodiments, the method includes multiplex PCR followed by an array for detection of the amplified products.

[0233] The multiplex-PCR methods of the invention can be in a variety of applications, such as genotyping, detection of chromosomal abnormalities (such as a fetal chromosome aneuploidy), gene mutation and polymorphism (such as single nucleotide polymorphisms, SNPs) analysis, gene deletion analysis, determination of paternity, analysis of genetic differences among populations, forensic analysis, measuring predisposition to disease, quantitative analysis of mRNA, and detection and identification of infectious agents (such as bacteria, parasite, and viruses). The multiplex PCR methods can also be used for non-invasive prenatal testing, such as paternity testing or the detection of fetal chromosome abnormalities.

Exemplary Primer Design Methods

[0234] Highly multiplexed PCR can often result in the production of a very high proportion of product DNA that results from unproductive side reactions such as primer dimer formation. In an embodiment, the particular primers that are most likely to cause unproductive side reactions may be removed from the primer library to give a primer library that will result in a greater proportion of amplified DNA that maps to the genome. The step of removing problematic primers, that is, those primers that are particularly likely to firm dimers has unexpectedly enabled extremely high PCR multiplexing levels for subsequent analysis by sequencing. In systems such as sequencing, where performance significantly degrades by primer dimers and/or other mischief products, greater than 10, greater than 50, and greater than 100 times higher multiplexing than other described multiplexing has been achieved. Note this is opposed to probe based detection methods, *e.g.* microarrays, TAQMAN, PCR etc. where an excess of primer dimers will not affect the outcome appreciably. Also note that the general belief in the art is that multiplexing PCR for sequencing is limited to about 100 assays in the same well. Fluidigm and Rain Dance offer platforms to perform 48 or 1000s of PCR assays in parallel reactions for one sample.

[0235] There are a number of ways to choose primers for a library where the amount of non-mapping primer dimer or other primer mischief products are minimized. Empirical data indicate that a small number of 'bad' primers are responsible for a large amount of non-mapping primer dimer side reactions. Removing these 'bad' primers can increase the percent of sequence reads that map to targeted loci. One way to identify the 'bad' primers is to look at the sequencing data

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of DNA that was amplified by targeted amplification; those primer dimers that are seen with greatest frequency can be removed to give a primer library that is significantly less likely to result in side product DNA that does not map to the genome. There are also publicly available programs that can calculate the binding energy of various primer combinations, and removing those with the highest binding energy will also give a primer library that is significantly less likely to result in side product DNA that does not map to the genome.

[0236] In some embodiments for selecting primers, an initial library of candidate primers is created by designing one or more primers or primer pairs to candidate target loci. A set of candidate target loci (such as SNPs) can selected based on publically available information about desired parameters for the target loci, such as frequency of the SNPs within a target population or the heterozygosity rate of the SNPs. In one embodiment, the PCR primers may be designed using the Primer3 program (the worldwide web at primer3.sourceforge.net; libprimer3 release 2.2.3, which is hereby incorporated by reference in its entirety). If desired, the primers can be designed to anneal within a particular annealing temperature range, have a particular range of GC contents, have a particular size range, produce target amplicons in a particular size range, and/or have other parameter characteristics. Starting with multiple primers or primer pairs per candidate target locus increases the likelihood that a primer or prime pair will remain in the library for most or all of the target loci. In one embodiment, the selection criteria may require that at least one primer pair per target locus remains in the library. That way, most or all of the target loci will be amplified when using the final primer library. This is desirable for applications such as screening for deletions or duplications at a large number of locations in the genome or screening for a large number of sequences (such as polymorphisms or other mutations) associated with a disease or an increased risk for a disease. If a primer pair from the library would produces a target amplicon that overlaps with a target amplicon produced by another primer pair, one of the primer pairs may be removed from the library to prevent interference.

[0237] In some embodiments, a score such as an "undesirability score" (higher score representing least desirability) is calculated (such as calculation on a computer) for most or all of the possible combinations of two primers from a library of candidate primers. In various embodiments, a score (such as an undesirability score) is calculated for at least 80, 90, 95, 98, 99, or 99.5% of the possible combinations of candidate primers in the library. Each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers. If desired, the score (such as the undesirability score) may also be

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based on one or more other parameters selected from the group consisting of heterozygosity rate of the target locus, disease prevalence associated with a sequence (*e.g.*, a polymorphism) at the target locus, disease penetrance associated with a sequence (*e.g.*, a polymorphism) at the target locus, specificity of the candidate primer for the target locus, size of the candidate primer, melting temperature of the candidate primer, melting temperature of the target amplicon, GC content of the target amplicon, GC content of the 3' end of the candidate primer, homopolymer length in the candidate primer, amplification efficiency of the target amplicon, size of the target amplicon, number of SNPs within the candidate primer, location of SNPs within the candidate primer, distance from an end of the amplicon to the target bases within the amplicon, and the number of target loci in an amplicon. In some embodiments, the lower the number of SNPs with the candidate primer (such as 2, 1 or 0 SNPs) the better. In some embodiments, there are no SNPs in the candidate primer. In some embodiments, SNPs (if any) are preferably not in the last 5 nucleotides in the 3' end of the candidate primer. In some embodiments, the target bases (the bases of interest in a target locus) are preferably near an end (the 3' or 5' end) of the amplicon; this may improve the quality of sequencing data (since bases near the end of an amplicon are sequenced more accurately), and/or allow shorter sequencing reads to be performed. In some embodiments, a single amplicon includes 2 or more target loci (such as 2 or more nearby SNPs or variants). In some embodiments, the specificity of the candidate primer for the target locus includes the likelihood that the candidate primer will mis-prime by binding and amplifying a locus other than the target locus it was designed to amplify. In some embodiments, one or more or all the candidate primers that mis-prime are removed from the library. In some embodiments to increase the number of candidate primers to choose from, candidate primers that may mis-prime are not removed from the library. In some embodiments, the optimal melting temperature for selection of the candidate primers is 57 °C. In some embodiments, the optimal size for selection of the candidate primers is a length of 24 nucleotides. In some embodiments, the optimal GC content for selection of the candidate primers is 50%. If multiple factors are considered, the score (such as the undesirability score) may be calculated based on a weighted average of the various parameters. The parameters may be assigned different weights based on their importance for the particular application that the primers will be used for. An exemplary score (such as an undesirability score) for a primer is shown below in which the parameters are weighted by various factors.

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score = (1)(total number of targets - number of targets covered) + (100)(number of SNPs in GC clamp) + (10)(number of SNPs in primer binding site) + (10)(number of similar primer pair designs) + (0.1)(distance of target base from amplicon end) + (0.1)(amplicon length) + (100) (interaction score)

where interaction score = max (-1 * ΔG value) as described herein

[0238] Another exemplary score for a primer is shown below.

score = (100)(number of SNPs in GC clamp) + (10)(number of SNPs in primer binding site) + (0.1)(distance of target base from amplicon end) + (0.1)(amplicon length) + (100) (interaction score)

where interaction score = max (-1 * ΔG value) as described herein

[0239] In some embodiments, the score for a primer pair is the worse score out of the scores for the two primers in the pair. An exemplary score (such as an undesirability score or the score in Example 20) for a pairs of designs (in which each design is one primer pair so that a pair of designs includes two primer pairs with a total of 4 primers) is shown below.

score = (10000000)(amplicon overlap) + (100)(distance between designs) + (1)(total number of targets - number of targets covered) + (100)(number of SNPs in GC clamp) + (10)(number of SNPs in primer binding site) + (10)(number of similar primer pair designs) + (0.1)(distance of target base from amplicon end) + (0.1)(amplicon length) + (100) (interaction score)

where interaction score = max (-1 * ΔG value) as described herein;

where amplicon overlap = overlap between the two amplicons formed by a pair of designs

[0240] In some embodiments, the score for a pair of designs is the worse score out of the scores for the four primers in the pair of designs.

[0241] In some embodiments, the primer with the highest score (such as the undesirability score) or any score representing least desirability is removed from the library. If the removed primer is a member of a primer pair that hybridizes to one target locus, then the other member of the

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primer pair may be removed from the library. The process of removing primers may be repeated as desired. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below a minimum threshold (such as any threshold for which the primers remaining in the library all have at least that level of desirability). In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number.

[0242] In various embodiments, after the score (such as the undesirability score) are calculated, the candidate primer that is part of the greatest number of combinations of two candidate primers with a score (such as an undesirability score) above a first minimum threshold (such as any threshold for which the primers remaining in the library all have at least that level of desirability) is removed from the library. This step ignores interactions equal to or below the first minimum threshold since these interactions are less significant. If the removed primer is a member of a primer pair that hybridizes to one target locus, then the other member of the primer pair may be removed from the library. The process of removing primers may be repeated as desired. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the first minimum threshold. If the number of candidate primers remaining in the library is higher than desired, the number of primers may be reduced by decreasing the first minimum threshold to a lower second minimum threshold (such as any threshold with a stricter cutoff than the first minimum threshold so that some of the least desirable primers are removed from the library) and repeating the process of removing primers. If the number of candidate primers remaining in the library is lower than desired, the method can be continued by increasing the first minimum threshold to a higher second minimum threshold (such as any threshold with a less strict cutoff than the first minimum threshold) and repeating the process of removing primers using the original candidate primer library, thereby allowing more of the candidate primers to remain in the library. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the second minimum threshold, or until the number of candidate primers remaining in the library is reduced to a desired number.

[0243] If desired, primer pairs that produce a target amplicon that overlaps with a target amplicon produced by another primer pair can be divided into separate amplification reactions.

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Multiple PCR amplification reactions may be desirable for applications in which it is desirable to analyze all of the candidate target loci (instead of omitting candidate target loci from the analysis due to overlapping target amplicons).

[0244] In various embodiments of any of the aspects of the invention, the selection method selects candidate primers and divides them into different pools (*e.g.*, 2, 3, 4, 5, 6, or more different pools). Each pool can be used to simultaneously amplify a large number of target loci (or a subset of target loci) in a single reaction volume. In some embodiments, a graph coloring algorithm is used to divide candidate primers into different pools. If desired, this method can be used to minimize the number of different pools needed to amplify most or all of the target loci.

[0245] In some embodiments, most or all of the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the target loci) are amplified by at least 2, 3, 4, 5, 6, or more different pools. In some embodiments, most or all of the bases in the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the bases in the target loci) are amplified by at least 2,3, 4, 5, 6, or more different pools. In some embodiments, most or all of the bases in the target loci (such as at least 70, 80, 90, 92, 94, 96, 98, 99, or 100% of the bases in the target loci) are amplified by at least 2, 3, 4, 5, 6, or more different primers or primer pairs in different pools. For example, a particular base in a target locus may be amplified by at least 2, 3, 4, 5, 6, or more different primers or primer pairs; wherein each different primer or primer pair is in a different pool. Using different primers or primer pairs to amplify each base allows multiple independent measurements of the base to be made, thereby increasing the accuracy of the method. Dividing the different primers or primer pairs that amplify the same base into different pools prevents interference due to overlapping amplicons being formed by different primers or primer pairs.

[0246] In one aspect, the invention features methods of selecting test primers from a library of candidate primers to form 2 or more different primer pools. In various embodiments, the selection involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing the candidate primer with the highest or worst score (such as an undesirability score) from the library of candidate primers; and (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a first pool. In some embodiments, the selection method is

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performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below a minimum threshold for the first pool. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In some embodiments, after the first pool is selected those primers are removed from further consideration and steps of the method (such as steps (ii) and (iii)) are repeated with the remaining primers to select a second pool. If desired, this method may be repeated to select the desired number of primer pools. In some embodiments, the score is based at least in part on the current coverage of the bases in the target locus (such as the number of other primer pools that have a primer or primer pair that amplifies a particular base in the target locus).

[0247] In one aspect, the invention features methods of selecting test primers from a library of candidate primers to form 2 or more different primer pools. In various embodiments, the selection of test primers are selected from a library of candidate primers involves (i) calculating on a computer a score (such as an undesirability score) for most or all of the possible combinations of two candidate primers from the library, wherein each score (such as an undesirability score) is based at least in part on the likelihood of dimer formation between the two candidate primers; (ii) removing from the library of candidate primers the candidate primer that is part of the greatest number of combinations of two candidate primers with a score (such as an undesirability score) above a first minimum threshold; (iii) if the candidate primer removed in step (ii) is a member of a primer pair, then removing the other member of the primer pair from the library of candidate primers; and (iv) optionally repeating steps (ii) and (iii), thereby selecting a first pool. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining in the library are all equal to or below the first minimum threshold for the first pool. In some embodiments, the selection method is performed until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In various embodiments, the selection method involves further reducing the number of candidate primers remaining in the library by decreasing the first minimum threshold used in step (ii) to a lower second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection method involves increasing the first minimum threshold used in step (ii) to a higher second minimum threshold and optionally repeating steps (ii) and (iii). In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primer combinations remaining

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in the library are all equal to or below the second minimum threshold, or until the number of candidate primers remaining in the library is reduced to a desired number for the first pool. In some embodiments, after the first pool is selected those primers are removed from further consideration and steps of the method (such as steps (ii) and (iii)) are repeated with the remaining primers to select a second pool. If desired, this method may be repeated to select the desired number of primer pools. In some embodiments, the score is based at least in part on the current coverage of the bases in the target locus (such as the number of other primer pools that have a primer or primer pair that amplifies a particular base in the target locus).

[0248] As discussed above, in some embodiments, a library is formed by starting with a library of candidate primers and removing primers until the primers remaining in the library have the desired characteristics for use as a final primer library.

[0249] In other embodiments, candidate primers are added to a library (such as a library starting with no primers) to form a library with the desired characteristics. In some embodiments, the candidate primer or primer pair with the most desirable score (such as the lowest undesirability score) is added to a library (such as a library starting with no primers). The process of adding candidate primers may be repeated as desired. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primers that have not been added to the library are all above a minimum threshold (such that all the candidate primers that have not been chosen for the library all have worse scores than the threshold). In some embodiments, the selection method is performed until the number of candidate primers that have been added to the library reaches a desired number. The library of selected primers can then be used in any of the methods of the invention.

[0250] In some embodiments, most (such as at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5%) or all of the possible sets of two primer pairs (two primer pairs with a total of 4 primers) are considered for inclusion in a library. In some embodiments, the set of two different candidate primer pair with the most desirable score (such as the lowest undesirability score) is added to a first pool (such as a first pool starting with no primers). In some embodiments, the set of two different candidate primer pairs with the next most desirable score is added to the first pool if it is connected to at most two sets of two different candidate primer pairs in the first pool. By "connected" for purposes of this step is meant that a single candidate primer pair in one set of two different candidate primer pairs is the same as a single candidate primer pair in another set of two different candidate primer pairs. If the set of two different candidate primer pairs is

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connected to more than two sets, it may be added to a different pool than the first pool. The process of set of two different candidate primer pair to pool(s) may be repeated as desired for the next set of two different candidate primer pairs with the next most desirable score. In some embodiments, the selection method is performed until the score (such as the undesirability score) for the candidate primers that have not been added to the pool(s) are all above a minimum threshold (such that all the candidate primers that have not been chosen for the pool(s) all have worse scores than the threshold). In some embodiments, the selection method is performed until the number of candidate primers that have been added to the pool(s) reaches a desired number. In some embodiments, the method involves storing designs in N number of doubly linked list data structures with the design pairs. N represents the current number of different primer pools. Initially, N =1, since there is only one primer pool. In some embodiments, a second pool is only created if necessary to include the desired target loci or the desired level of coverage of target loci. The library of selected primers can then be used in any of the methods of the invention.

[0251] In some embodiments, the minimum threshold, the first minimum threshold, or the second minimum threshold is an interaction score equal to or about 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 kcal/mol. In some embodiments, the interaction score is calculated as followed as described further herein:

Interaction score = max (-1 * ΔG value); or

interaction_score = max(- deltaG_2, 0.8 * (- deltaG_1))

where

deltaG_2 = Gibbs energy (energy required to break the dimer) for a dimer that is extensible by PCR on both ends, *i.e,*. the 3' end of each primer anneals to the other primer; and

deltaG_1 = Gibbs energy for a dimer that is extensible by PCR on at least one end.

[0252] These selection methods minimize the number of candidate primers that have to be removed from the library to achieve the desired reduction in primer dimers. By removing a smaller number of candidate primers from the library, more (or all) of the target loci can be amplified using the resulting primer library.

[0253] Multiplexing large numbers of primers imposes considerable constraint on the assays that can be included. Assays that unintentionally interact result in spurious amplification

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products. The size constraints of miniPCR may result in further constraints. In an embodiment, it is possible to begin with a very large number of potential SNP targets (between about 500 to greater than 1 million) and attempt to design primers to amplify each SNP. Where primers can be designed it is possible to attempt to identify primer pairs likely to form spurious products by evaluating the likelihood of spurious primer duplex formation between all possible pairs of primers using published thermodynamic parameters for DNA duplex formation. Primer interactions may be ranked by a scoring function related to the interaction and primers with the worst interaction scores are eliminated until the number of primers desired is met. In cases where SNPs likely to be heterozygous are most useful, it is possible to also rank the list of assays and select the most heterozygous compatible assays. Experiments have validated that primers with high interaction scores are most likely to form primer dimers. At high multiplexing it is not possible to eliminate all spurious interactions, but it is essential to remove the primers or pairs of primers with the highest interaction scores *in silico* as they can dominate an entire reaction, greatly limiting amplification from intended targets. We have performed this procedure to create multiplex primer sets of up to and in some cases more than 10,000 primers. The improvement due to this procedure is substantial, enabling amplification of more than 80%, more than 90%, more than 95%, more than 98%, and even more than 99% on target products as determined by sequencing of all PCR products, as compared to 10% from a reaction in which the worst primers were not removed. When combined with a partial semi-nested approach as previously described, more than 90%, and even more than 95% of amplicons may map to the targeted sequences.

[0254] Note that there are other methods for determining which PCR probes are likely to form dimers. In an embodiment, analysis of a pool of DNA that has been amplified using a non-optimized set of primers may be sufficient to determine problematic primers. For example, analysis may be done using sequencing, and those dimers which are present in the greatest number are determined to be those most likely to form dimers, and may be removed.

[0255] This method has a number of potential application, for example to SNP genotyping, heterozygosity rate determination, copy number measurement, and other targeted sequencing applications. In an embodiment, the method of primer design may be used in combination with the mini-PCR method described elsewhere in this document. In some embodiments, the primer design method may be used as part of a massive multiplexed PCR method.

[0256] The use of tags on the primers may reduce amplification and sequencing of primer dimer products. In some embodiments, the primer contains an internal region that forms a loop

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structure with a tag. In particular embodiments, the primers include a 5' region that is specific for a target locus, an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. In some embodiments, the loop region may lie between two binding regions where the two binding regions are designed to bind to contiguous or neighboring regions of template DNA. In various embodiments, the length of the 3' region is at least 7 nucleotides. In some embodiments, the length of the 3' region is between 7 and 20 nucleotides, such as between 7 to 15 nucleotides, or 7 to 10 nucleotides, inclusive. In various embodiments, the primers include a 5' region that is not specific for a target locus (such as a tag or a universal primer binding site) followed by a region that is specific for a target locus, an internal region that is not specific for the target locus and forms a loop structure, and a 3' region that is specific for the target locus. Tag-primers can be used to shorten necessary target-specific sequences to below 20, below 15, below 12, and even below 10 base pairs. This can be serendipitous with standard primer design when the target sequence is fragmented within the primer binding site or, or it can be designed into the primer design. Advantages of this method include: it increases the number of assays that can be designed for a certain maximal amplicon length, and it shortens the "non-informative" sequencing of primer sequence. It may also be used in combination with internal tagging (see elsewhere in this document).

[0257] In an embodiment, the relative amount of nonproductive products in the multiplexed targeted PCR amplification can be reduced by raising the annealing temperature. In cases where one is amplifying libraries with the same tag as the target specific primers, the annealing temperature can be increased in comparison to the genomic DNA as the tags will contribute to the primer binding. In some embodiments we are using considerably lower primer concentrations than previously reported along with using longer annealing times than reported elsewhere. In some embodiments the annealing times may be longer than 3 minutes, longer than 5 minutes, longer than 8 minutes, longer than 10 minutes, longer than 15 minutes, longer than 20 minutes, longer than 30 minutes, longer than 60 minutes, longer than 120 minutes, longer than 240 minutes, longer than 480 minutes, and even longer than 960 minutes. In an embodiment, longer annealing times are used than in previous reports, allowing lower primer concentrations. In various embodiments, longer than normal extension times are used, such as greater than 3, 5, 8, 10, or 15 minutes. In some embodiments, the primer concentrations are as low as 50 nM, 20 nM, 10 nM, 5 nM, 1 nM, and lower than 1 uM. This surprisingly results in robust performance for highly multiplexed reactions, for example 1,000-plex reactions, 2,000-

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plex reactions, 5,000-plex reactions, 10,000-plex reactions, 20,000-plex reactions, 50,000-plex reactions, and even 100,000-plex reactions. In an embodiment, the amplification uses one, two, three, four or five cycles run with long annealing times, followed by PCR cycles with more usual annealing times with tagged primers.

[0258] To select target locations, one may start with a pool of candidate primer pair designs and create a thermodynamic model of potentially adverse interactions between primer pairs, and then use the model to eliminate designs that are incompatible with other the designs in the pool.

[0259] In an embodiment, the invention features a method of decreasing the number of target loci (such as loci that may contain a polymorphism or mutation associated with a disease or disorder or an increased risk for a disease or disorder such as cancer) that need to be detected for a diagnosis and/or increasing the disease load that is detected (*e.g.*, increasing the number of polymorphisms or mutations that are detected). In some embodiments, the method includes ranking (such as ranking from highest to lowest) loci by frequency or reoccurrence of a polymorphism or mutation (such as a single nucleotide variation, insertion, or deletion, or any of the other variations described herein) in each locus among subjects with the disease or disorder such as cancer. In some embodiments, PCR primers are designed to some or all of the loci. During selection of PCR primers for a library of primers, primers to loci that have a higher frequency or reoccurrence (higher ranking loci) are favored over those with a lower frequency or reoccurrence (lower ranking loci). In some embodiments, this parameter is included as one of the parameters in the calculation of the scores (such as the undesirability scores) described herein. If desired, primers (such as primers to high ranking loci) that are incompatible with other designs in the library can be included in a different PCR library/pool. In some embodiments, multiple libraries/pools (such as 2, 3, 4, 5 or more) are used in separate PCR reactions to enable amplification of all (or a majority) of the loci represented by all the libraries/pools. In some embodiment, this method is continued until sufficient primers are included in one or more libraries/pools such that the primers, in aggregate, enable the desired disease load to be captured for the disease or disorder (*e.g.*, such as by detection of at least 80, 85, 90, 95, or 99% of the disease load).

[0260] In some embodiments, the library of candidate primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers or different primer pairs. In some embodiments, only a relatively small number of candidate primers need to be removed

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from the library to achieve the desired reduction in primer dimers. In some embodiments, less than 30, 20, 15, 10, 5, or 2% of the candidate primers are removed from the library prior to use of the resulting library for multiplex PCR amplification of target loci. In some embodiments, a relatively large number of candidate primers are removed from the library to achieve the desired characteristics for the resulting library. In some embodiments, at least 20, 30, 40, 50, 60, 70, 80, or 90% of the candidate primers are removed from the library prior to use of the resulting library for multiplex PCR amplification of target loci. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers or different primer pairs remain in the library (after removal of some candidate primers from the library).

[0261] After the selection process, the primers remaining in the library may be used in any of the methods of the invention.

Exemplary Methods for Determining Interaction Scores

[0262] Exemplary methods of determining a ΔG value (such as the change in Gibbs free energy for primer dimer formation) or an interaction score that indicates the likelihood of dimer formation between candidate primers are described below. In some embodiments, a thermodynamic Nearest-Neighbors approach is used to calculate the likelihood of dimer formation between any two primers (*see, e.g.*, Rahmann and Grafe (2004), "Mean and variance of the Gibbs free energy of oligonucleotides in the nearest neighbor model under varying conditions" Bioinformatics 20, 2928-2933; Allawi, H. T. & SantaLucia, J., Jr. (1998), "Thermodynamics of Internal C-T Mismatches in DNA", *Nucleic Acids Res. 26*, 2694-2701; Peyret, N., Seneviratne, P. A., Allawi, H. T. & SantaLucia, J., Jr. (1999), "Nearest-Neighbor Thermodynamics and NMR of DNA Sequences with Internal A-A, C-C, G-G, and T-T Mismatches", *Biochemistry 38*, 3468-3477; Allawi, H. T. & SantaLucia, J., Jr. (1998), "Nearest-Neighbor Thermodynamics of Internal A-C Mismatches in DNA: Sequence Dependence and pH Effects", *Biochemistry 37*, 9435-9444.; Allawi, H. T. & SantaLucia, J., Jr. (1998), "Nearest Neighbor Thermodynamic Parameters for Internal G-A Mismatches in DNA", *Biochemistry 37*, 2170-2179; and Allawi, H. T. & SantaLucia, J., Jr. (1997), "Thermodynamics and NMR of Internal G-T Mismatches in DNA", *Biochemistry 36*, 10581-10594; MultiPLX 2.1 (Kaplinski L, Andreson R, Puurand T, Remm M. MultiPLX: automatic grouping and evaluation of PCR

primers. Bioinformatics. 2005 Apr 15;21(8):1701-2, which are each hereby incorporated by reference in its entirety).

[0263] In some embodiments, the following steps are performed.

Step 1

[0264] For each primer in a set of candidate primers, compare to every other candidate primer in the following way. Implement an ungapped thermodynamic alignment between the two primers, matching the 5' end of the first primer to the 3' end of the second primer. Taking the first two bases (dinucleotide) that align to the other primer and vice versa, determine the ΔH and ΔS values for the dinucleotide in one primer hybridizing to the dinucleotide in the other primer (see the "AT" hybridizing to "GA" in FIG. 33A). ΔH and ΔS values for various combinations of dinucleotides are known and can be determined, for example, using a thermodynamic look up table (such as the Unified NN model parameters according to Allawi and SantaLucia (1997) "Thermodynamics and NMR of internal G-T mismatches in DNA". *Biochemistry,* 36: 10581-10594, which is hereby incorporated by reference in its entirety). Use the ΔH and ΔS values to calculate ΔG for that interaction as follows or as described in any known equation for this.

ΔG = (1000.0 * ΔH - (temperature * (ΔS + 0.368 * (numPhosphates/2) *log(saltConcentration)))) / 1000.0

[0265] In some embodiments, one or more of the following conditions are assumed for this calculation: temperature: of 60.0 °C, primer concentration of 100 nM, or salt concentration of 100 mM. In some embodiments, other conditions are assumed for this calculation, such as the conditions that will be used for multiplex PCR with the pool. Iterate over the remainder of the primer that aligns with the other primer by sliding the bases being observing one base to the right. Continue until ΔG has been calculated for all dinucleotides that align (FIG. 33B). The ΔG for that alignment of the primer pair is the sum of the ΔG values for the various dinucleotides.

Step 2

[0266] Shift the alignment of the two primers (FIGs. 33C and 33D).

Step 3

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[0267] Repeat step 1 again for the new alignment (FIGs. 33E and 33F).

Step 4

[0268] After aligning all possible alignments between the two primers, determine the final ΔG value and the interaction score.

[0269] In some embodiments, the ΔG value for a combination of primers is the lowest ΔG value (the lowest numerical value, which is indicative of the greatest likelihood of primer dimer formation) out of the ΔG values for all possible alignments between the two primers. For example, if one alignment has a ΔG value of -12 kcal/mol and another alignment has a ΔG value of -2 kcal/mol then -12 kcal/mol (worse value) is used as the ΔG value for that combination of primers.

[0270] For algorithms such as the one in Example 16 in which it is easiest to rank primers based on assigning the worse combination of primers (those with the greatest likelihood of dimer formation) the highest interaction score, then the interaction score can be calculated as follows.

Interaction score = max (-1 * ΔG value)

[0271] For example, if one alignment has a ΔG value of -12 kcal/mol and another alignment has a ΔG value of -2 kcal/mol, then 12 kcal/mol is used as the interaction score. In this case, the interaction score with the largest numerical positive number indicates the least desirable combination of primers due to the greatest likelihood of primer dimer formation.

[0272] In some embodiments, the interaction score is calculated as follows (this score weights the ΔG values depending on the number of ends that a dimer can be extended by PCR).

interaction_score = max(- deltaG_2, 0.8 * (- deltaG_1))

where

deltaG_2 = Gibbs energy (energy required to break the dimer) for a dimer that is extensible by PCR on both ends, *i.e,.* the 3' end of each primer anneals to the other primer; and

deltaG_1 = Gibbs energy for a dimer that is extensible by PCR on at least one end.

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[0273] In some embodiments, deltaG_2 is determined by performing steps 1-4 above for all the alignments in which a dimer is extensible by PCR on both ends. The deltaG_2 value is the lowest ΔG value (the lowest numerical value, which is indicative of the greatest likelihood of primer dimer formation) for all the alignments in which a dimer is extensible by PCR on both ends.

[0274] In some embodiments, deltaG_1 is determined by performing steps 1-4 above for all the alignments in which a dimer is extensible by PCR on at least one end (such as by PCR on one end or by PCR on both ends). The deltaG_1 value is the lowest ΔG value (the lowest numerical value, which is indicative of the greatest likelihood of primer dimer formation) for all the alignments in which a dimer is extensible by PCR on at least one end.

[0275] In some embodiments, possible loop structures or gaps in alignment between primers are also considered.

[0276] In some embodiments, ΔG values from step 4 for each possible combination of two primers (each possible primer dimer) in a library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol. In some embodiments, ΔG values from step 4 for at least 80, 85, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library for possible combinations of that primer with other primers in the library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol. In some embodiments, possible combinations of two primers in a library that have positive ΔG values are ignored since these values are indicative of no likelihood to for primer dimers. In some embodiments for the possible combination of two primers in a library that have negative ΔG values, the ΔG values are between -20 and 0 kcal/mol, such as between -15 and 0 kcal/mol, -10 and 0 kcal/mol, -8 and 0 kcal/mol, -7 and 0 kcal/mol, -6 and 0 kcal/mol, -5.5 and 0 kcal/mol, -5 and 0 kcal/mol, -4.5 and 0 kcal/mol, -4 and 0 kcal/mol, -3.5 and 0 kcal/mol, -3 and 0 kcal/mol, -2.5 and 0 kcal/mol, -2 and 0 kcal/mol, or -1.5 and 0 kcal/mol, inclusive.

[0277] In some embodiments, the interaction scores from step 4 for each possible combination of two primers in a library are all equal to or less than 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 kcal/mol. In some embodiments, the interaction scores from step 4 for at least 80, 85, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library for possible combinations of that primer with other primers in the library are all equal to or less than 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 kcal/mol. In some embodiments, possible combination of two primers in a library that have negative interaction scores are ignored since these values are indicative of no

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likelihood to for primer dimers. In some embodiments for the possible combination of two primers in a library that have positive interaction scores, the interaction scores are between 20 and 0 kcal/mol, such as between 15 and 0 kcal/mol, 10 and 0 kcal/mol, 8 and 0 kcal/mol, 7 and 0 kcal/mol, 6 and 0 kcal/mol, 5.5 and 0 kcal/mol, 5 and 0 kcal/mol, 4.5 and 0 kcal/mol, 4 and 0 kcal/mol, 3.5 and 0 kcal/mol, 3 and 0 kcal/mol, 2.5 and 0 kcal/mol, 2 and 0 kcal/mol, or 1.5 and 0 kcal/mol, inclusive.

[0278] In some embodiments, the score (such as the undesirability score) for candidate primers is based at least in part on the ΔG value or the interaction score that indicates the likelihood of dimer formation between candidate primers as calculated using any of these methods.

Exemplary Primer Libraries

[0279] In one aspect, the invention features libraries of primers, such as primers selected from a library of candidate primers using any of the methods of the invention. In some embodiments, the library includes primers that simultaneously hybridize (or are capable of simultaneously hybridizing) to or that simultaneously amplify (or are capable of simultaneously amplifying) at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci in one reaction volume. In various embodiments, the library includes primers that simultaneously amplify (or are capable of simultaneously amplifying) between 1,000 to 2,000; 2,000 to 5,000; 5,000 to 7,500; 7,500 to 10,000; 10,000 to 20,000; 20,000 to 25,000; 25,000 to 30,000; 30,000 to 40,000; 40,000 to 50,000; 50,000 to 75,000; or 75,000 to 100,000 different target loci in one reaction volume, inclusive. In various embodiments, the library includes primers that simultaneously amplify (or are capable of simultaneously amplifying) between 1,000 to 100,000 different target loci in one reaction volume, such as between 1,000 to 50,000; 1,000 to 30,000; 1,000 to 20,000; 1,000 to 10,000; 2,000 to 30,000; 2,000 to 20,000; 2,000 to 10,000; 5,000 to 30,000; 5,000 to 20,000; or 5,000 to 10,000 different target loci, inclusive. In some embodiments, the library includes primers that simultaneously amplify (or are capable of simultaneously amplifying) the target loci in one reaction volume such that less than 60, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.5% of the amplified products are primer dimers. The various embodiments, the amount of amplified products that are primer dimers is between 0.5 to 60%, such as between 0.1 to 40%, 0.1 to 20%, 0.25 to 20%, 0.25 to 10%, 0.5 to 20%, 0.5 to

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10%, 1 to 20%, or 1 to 10%, inclusive. In some embodiments, the primers simultaneously amplify (or are capable of simultaneously amplifying) the target loci in one reaction volume such that at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the amplified products are target amplicons. In various embodiments, the amount of amplified products that are target amplicons is between 50 to 99.5%, such as between 60 to 99%, 70 to 98%, 80 to 98%, 90 to 99.5%, or 95 to 99.5%, inclusive. In some embodiments, the primers simultaneously amplify (or are capable of simultaneously amplifying) the target loci in one reaction volume such that at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified. In various embodiments, the amount target loci that are amplified is between 50 to 99.5%, such as between 60 to 99%, 70 to 98%, 80 to 99%, 90 to 99.5%, 95 to 99.9%, or 98 to 99.99% inclusive. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 primer pairs, wherein each pair of primers includes a forward test primer and a reverse test primer where each pair of test primers hybridize to a target locus. In some embodiments, the library of primers includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 individual primers that each hybridize to a different target locus, wherein the individual primers are not part of primer pairs.

[0280] In some embodiments, the library includes primers (such as at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers or different primer pairs) that simultaneously amplify (or are capable of simultaneously amplifying) the target loci (such as at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci) in one reaction volume such that one or more of the following: (i) less than 60, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.5% of the amplified products are primer dimers, (ii) at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the amplified products are target amplicons, (iii) at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified, (iv) at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold, (v) at least 60, 70, 80, 90, 95, 96, 97, 98, 99,

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99.5, or 100% of the amplified products map to the human genome, or (vi) any combination thereof.

[0281] In some embodiments, the library includes at least 1,000 different primers or different primer pairs (such as at least 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers or different primer pairs) that simultaneously amplify (or are capable of simultaneously amplifying) at least 1,000 different target loci (such as at least 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci) in one reaction volume such that one or more of the following: (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments for the amplification of nucleic acids (such as DNA or RNA) from a single cell (such as a fetal or embryonic cell), the library includes at least 1,000 different primers or different primer pairs (such as at least 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers or different primer pairs) that simultaneously amplify (or are capable of simultaneously amplifying) at least 1,000 different target loci (such as at least 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci) in one reaction volume such that one or more of the following: (i) less than 60% of the amplified products are primer dimers and at least 10% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 10% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 10% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 10% of the amplified products are target amplicons, (v) less than 5% of the amplified products are primer dimers and at least 15% of the amplified products are target amplicons; (vi) less than 60% of the amplified products are primer dimers and at least 20% of the amplified products are target amplicons, (vii) less than 40% of the amplified products are primer dimers and at least 20% of the amplified products are

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target amplicons, (viii) less than 20% of the amplified products are primer dimers and at least 20% of the amplified products are target amplicons, (ix) less than 10% of the amplified products are primer dimers and at least 20% of the amplified products are target amplicons, (x) less than 5% of the amplified products are primer dimers and at least 20% of the amplified products are target amplicons; (xi) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (xii) less than 40% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (xiii) less than 20% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (xiv) less than 10% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (xv) less than 5% of the amplified products are primer dimers and at least 45% of the amplified products are target amplicons; (xvi) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (xvii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (xviii) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (xviiii) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, one or more of these embodiments (*e.g.*, percent of primer dimers, target amplicons, or amplified target loci) is achieved after greater than or equal to 5, 10, 20, 30, 40, 50, or 60 cycles of PCR are performed. In some embodiments for a library that amplifies human target loci, at least 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the amplified products map to the human genome.

[0282] In various embodiments, the concentration of each primer is less than 100, 75, 50, 25, 20, 10, 5, 2, 1, 0.5, 0.1, or 0.05 nM, or less than 500, 100, 10, or 1 uM. In various embodiments, the concentration of each primer is between 1 uM to 100 nM, such as between 1 uM to 1 nM, 1 to 75 nM, 1 to 20 nM, 1 to 10 nM, 2 to 50 nM or 5 to 50 nM, inclusive. In some embodiments, the concentration of one or more universal primers is between 0.2 to 3 μM, such as between 0.5 and 2.5 μM, 0.5 to 1 μM, or 1 to 2.5 μM per primer, inclusive, and the concentration of each primer except the universal primer(s) is between 1 uM to 100 nM, such as between 1 uM to 1 nM, 1 to 75 nM, 1 to 20 nM, 1 to 10 nM, 2 to 50 nM or 5 to 50 nM, inclusive. In various embodiments, the GC content of the primers is between 30 to 80%, such as between 20 to 70%,

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40 to 70%, or 50 to 60%, inclusive. In some embodiments, the range of GC content of the primers is less than 30, 20, 10, or 5%. In some embodiments, the range of GC content of the primers is between 5 to 30%, such as 5 to 20% or 5 to 10%, inclusive. In some embodiments, there is a high GC content in the 3' end of the primers. In some embodiments, there are at least 2 (such as 3, 4, or 5) guanines or cytosines in the last 5 bases at the 3' end of the primers. In some embodiments, there are at least 1 (such as 2 or 3) guanines or cytosines in the last 3 bases at the 3' end of the primers. In some embodiments, a maximum of 2 (such as 2, 1, or 0) bases in the last 5 bases at the 3' end of the primers are guanines or cytosines. In some embodiments, a maximum of 1 (such as 1 or 0) base in the last 5 bases at the 3' end of the primers is a guanine or cytosine. In some embodiments, the maximum length of a homopolymer (the same base in a row) in the primers is 12, 10, 8, 6, 5, 4, 3, or 2 consecutive nucleotides. In some embodiments, the melting temperature (T_m) of the test primers is between 40 to 80 °C, such as 50 to 70 °C, 55 to 65 °C, 54 to 60.5 °C, or 57 to 60.5 °C, inclusive. In some embodiments, the T_m is calculated using the Primer3 program (libprimer3 release 2.2.3) using the built-in SantaLucia parameters (the World Wide Web at primer3.sourceforge.net). In some embodiments, the range of melting temperature of the primers is less than 15, 10, 5, 3, or 1 °C. In some embodiments, the range of melting temperature of the primers is between 1 to 15 °C, such as between 1 to 10 °C, 1 to 5 °C, or 1 to 3 °C, inclusive. In some embodiments, the range of melting temperatures of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the primers is between 1 to 15 °C, such as between 1 to 10 °C, 1 to 5 °C, 1 to 3 °C, 2 to 5 °C, 3 to 10 °C, or 3 to 5 °C, inclusive. In some embodiments, the length of the primers is between 15 to 100 nucleotides, such as between 15 to 75 nucleotides, 15 to 40 nucleotides, 17 to 35 nucleotides, 18 to 30 nucleotides, 20 to 65 nucleotides, inclusive. In some embodiments, the range of the length of the primers is less than 50, 40, 30, 20, 10, or 5 nucleotides. In some embodiments, the range of the length of the primers is between 5 to 50 nucleotides, such as 5 to 40 nucleotides, 5 to 20 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides, inclusive. In some embodiments, the length of the target amplicons is between 30 and 400 nucleotides, such as between 30 and 200 nucleotides, or 100 and 400 nucleotides, inclusive. In some embodiments, the length of the target amplicons is at least 100; 200; 300; 400; 500; 600; 700; 800; 900; 1,000; 1,200; 1,500; 2,000; or 3,000 nucleotides. In some

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embodiments, the length of the target amplicons is between 100 and 1,500 nucleotides, such as between 100 to 1,000; 100 to 500, 500 to 750, or 750 to 1,000 nucleotides, inclusive. Longer amplicons may be desirable, *e.g.,* for applications in which is it desirable to screen for multiple potential mutations in one amplicon, such as carrier screening. In some embodiments, one round of PCR is performed to produce relatively long amplicons (such as at least 250 or 500 nucleotides in length) and then a second round of PCR is performed to produce shorter amplicons (to amplify regions within the amplicons amplified in the first round of PCR, such as regions of less than 200 or 100 nucleotides in length). In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 or all of the target amplicons have a length that falls within the range of the average length of the amplicons ± 5% of the average length, average length ± 20%, average length ± 20%, or average length ± 30%, or average length ± 50%.

[0283] In some embodiments, library includes at least at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers that each includes a target specific sequence, such as a sequence that binds a target locus but does not substantially bind to other nucleic acids (such as non-target loci) in a sample, *e.g.*, a biological sample, which naturally includes other nucleic acids. In some embodiments, each primer binds and amplifies a target locus by at least 2, 4, 6, 8, 10, 20, 50-fold or more than one or more (or all) other nucleic acids (such as non-target loci) in a sample. In some embodiments, the library includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target specific primers (*e.g.,* primers that are specific for a target locus). In some embodiments, part or all of the polynucleotide sequence is a non-random sequence for at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different primers in the library. In some embodiments, library also includes a universal primer, a random primer, and a primer with a non-naturally occurring polynucleotide sequence, or a primer with a polynucleotide sequence not naturally

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found in a human in some embodiments, the universal or random primer has a non-naturally occurring polynucleotide sequence or a polynucleotide sequence not naturally found in a human.

[0284] In some embodiments, the composition includes at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with a polynucleotide sequence of a human nucleic acid and at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) with a polynucleotide sequence that is not found in a human (such as a universal primer, a primer that comprises a region or consists entirely of random nucleotides, or a primer with a region such as a tag or barcode of one or more nucleotides that are not found in a human or are not found in nature as part of the polynucleotide sequence of the primer). In some embodiments, at least one primer (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers) includes a region of one or more nucleotides that is not naturally part of the primer sequence (such as a region added to the 5' end of the target specific sequence in the primer or an internal region added between the 5' and 3' ends of the primer). In some embodiments, the primer is free of the nucleic acids (such as genes) which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. In some embodiments, the primer has been separated from one or more components that naturally accompany the corresponding sequence in nature (such as in the genome of a human). Typically, each primer is at least 90, 95, 98, 99, 99.9, or 100%, by weight, free from the molecules (such as proteins, nucleic acids, and naturally-occurring organic molecules) that naturally accompany the corresponding sequence in nature (such as in the genome of a human). Purity can be assayed by any appropriate method, *e.g.*, by electrophoresis or HPLC analysis.

[0285] In some embodiments, the primers in the library are not immobilized (such as not immobilized to a solid support) or not part of a microarray. In some embodiments, the primers are dissolved in solution (such as dissolved in the liquid phase). In some embodiments, the library comprises a microarray. In some embodiments, the amplified products are detected using an array, such as an array with probes to one or more chromosomes of interest (*e.g.*, chromosome 13, 18, 21, X, Y, or any combination thereof).

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[0286] In some embodiments, at least one of the primers (such as at least 20, 40, 80, 90, 95, 98, 99, 99.5, or 100% of the primers) in a library are nucleic acid analogs that have a lower likelihood of primer dimerization compared to the naturally-occurring nucleic acids (*see, e.g.,* US Patent Nos. 7,414,118 and 6,001,611; which are each hereby incorporated by reference in its entirety). Exemplary nucleic acid analogs have a modified pyrimidine nucleobase, or a purine or pyrimidine base that contains an exocyclic amine.

[0287] In some embodiments, the primer library includes a small number of primers (such as less than 5, 2, 1, or 0.5% of the primers in the library) that do not have one or more of the properties described herein. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have one or more of the following properties: (i) ΔG values for possible combinations of that primer with other primers in the library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol; (ii) ΔG values for the possible combination of that primer with other primers in the library that have negative ΔG are between -20 and 0 kcal/mol, such as between -15 and 0 kcal/mol, -10 and 0 kcal/mol, -8 and 0 kcal/mol, -7 and 0 kcal/mol, -6 and 0 kcal/mol, -5.5 and 0 kcal/mol, -5 and 0 kcal/mol, -4.5 and 0 kcal/mol, -4 and 0 kcal/mol, -3.5 and 0 kcal/mol, -3 and 0 kcal/mol, -2.5 and 0 kcal/mol, -2 and 0 kcal/mol, or -1.5 and 0 kcal/mol, inclusive; (iii) the GC content is between 30 to 80%, such as between 20 to 70%, 40 to 70%, or 50 to 60%, inclusive; (iv) the range of GC content is less than 30, 20, 10, or 5% or the range of GC content of the primers is between 5 to 30%, such as 5 to 20%, or 5 to 10%, inclusive; (v) a maximum of 2 (such as 2, 1, or 0) bases in the last 5 bases at the 3' end of the primers are guanines or cytosines; (vi) the melting temperature (T_m) of the primers is between 40 to 80 °C, such as 50 to 70 °C, 55 to 65 °C, 54 to 60.5 °C, or 57 to 60.5 °C, inclusive; (vii) the range of melting temperature of the primers is less than 15, 10, 5, 3, or 1 °C; (viii) the range of melting temperature of the primers is between 1 to 15 °C, such as between 1 to 10 °C, 1 to 5 °C, 1 to 3 °C, 2 to 5 °C, 3 to 10 °C, or 3 to 5 °C, inclusive; (ix) the length of the primers is between 15 to 100 nucleotides, such as between 15 to 75 nucleotides, 15 to 40 nucleotides, 17 to 35 nucleotides, 18 to 30 nucleotides, or 20 to 65 nucleotides, inclusive; (x) the range of the length of the primers is less than 50, 40, 30, 20, 10, or 5 nucleotides; (xi) the range of the length of the primers is between 5 to 50 nucleotides, such as 5 to 40 nucleotides, 5 to 20 nucleotides, or 5 to 10 nucleotides, (xii) the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides; (xiii) the length of the target amplicons is between 30 and 400 nucleotides, such as between 30 and 200

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nucleotides, or 100 and 400 nucleotides; (xiv) the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides; (xv) the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides; (xvi) the maximum length of a homopolymer in the primers is 12, 10, 8, 6, 5, 4, 3, or 2 consecutive nucleotides; (xvii) the concentration of each primer is less than 100, 75, 50, 25, 20, 10, 5, 2, 1, 0.5, 0.1, or 0.05 nM, or less than 500, 100, 10, or 1 uM; (xviii) the concentration of each primer is between 1 uM to 100 nM, such as between 1 uM to 1 nM, 1 to 75 nM, 1 to 20 nM, 1 to 10 nM, 2 to 50 nM, or 5 to 50 nM, inclusive; (xix) at least 80, 90, 92, 94, 96, 98, 99, or 100% of the molecules of that primer are extended to form amplified products; (xx) SNPs (if any) are not in the last 5 nucleotides in the 3' end of the candidate primer; (xxi) the target bases (the bases of interest in a target locus) are near an end (the 3' or 5' end) of the amplicon; (xxii) the region of hybridization is separated from the polymorphic site by a small number of bases, where the small number is selected from the group consisting of 1, 2, 3, 4, 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, 26 to 30, and 31 to 60; (xxiii) the length of the annealing step is greater than 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes, (xxiv) the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive, (xxv) the length of the annealing step is greater than 5 minutes (such greater than 10, or 15 minutes), and the concentration of each primer is less than 20 nM, (xxvi) the length of the annealing step is greater than 5 minutes (such greater than 10, or 15 minutes), and the concentration of each primer is between 1 to 20 nM, or 1 to 10 nM, inclusive; (xxvii) the length of the annealing step is greater than 20 minutes (such as greater than 30, 45, 60, or 90 minutes), and the concentration of each primer is less than 1 nM; (xxviii) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers; (xxix) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers; (xxx) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature of the primers; (xxxi) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature of the primers, (xxxii) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the average

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melting temperature of the primers; and (xxviii) any combination thereof. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have one or more of the following properties: (i) ΔG values for possible combinations of that primer with other primers in the library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol; (ii) ΔG values for the possible combination of that primer with other primers in the library that have negative ΔG are between -20 and 0 kcal/mol, such as between -15 and 0 kcal/mol, -10 and 0 kcal/mol, -8 and 0 kcal/mol, -7 and 0 kcal/mol, -6 and 0 kcal/mol, -5.5 and 0 kcal/mol, -5 and 0 kcal/mol, -4.5 and 0 kcal/mol, -4 and 0 kcal/mol, -3.5 and 0 kcal/mol, -3 and 0 kcal/mol, -2.5 and 0 kcal/mol, -2 and 0 kcal/mol, or -1.5 and 0 kcal/mol, inclusive; (iii) the melting temperature (T_m) of the primers is between 40 to 80 °C, such as 50 to 70 °C, 55 to 65 °C, 54 to 60.5 °C, or 57 to 60.5 °C, inclusive; (iv) the range of melting temperature of the primers is less than 15, 10, 5, 3, or 1 °C; (v) the range of melting temperature of the primers is between 1 to 15 °C, such as between 1 to 10 °C, 1 to 5 °C, 1 to 3 °C, 2 to 5 °C, 3 to 10 °C, or 3 to 5 °C, inclusive; (vi) the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides; (vii) the length of the target amplicons is between 30 and 400 nucleotides, such as between 30 and 200 nucleotides, or 100 and 400 nucleotides; (viii) the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides; (ix) the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides; (x) the concentration of each primer is less than 100, 75, 50, 25, 20, 10, 5, 2, 1, 0.5, 0.1, or 0.05 nM, or less than 500, 100, 10, or 1 uM; (xi) the concentration of each primer is between 1 uM to 100 nM, such as between 1 uM to 1 nM, 1 to 75 nM, 1 to 20 nM, 1 to 10 nM, 2 to 50 nM, or 5 to 50 nM, inclusive; (xii) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers; (xiii) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers; (xiv) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature of the primers; (xv) the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature of the primers, (xvi) the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to

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5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the average melting temperature of the primers; and (xvii) any combination thereof.

[0288] In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have one or more of the following properties: (i) ΔG values for possible combinations of that primer with other primers in the library are all equal to or greater than -10 kcal/mol, (ii) the range of melting temperature of the primers is between 1 to 15 °C, (iii) the length of the target amplicons is between 50 and 100 nucleotides, (iv) the concentration of each primer is less than 20 nM, (v) the length of the annealing step is greater than 5 minutes (such as greater than 10 minutes), (vi) the length of the annealing step is greater than 5 minutes (such greater than 10 minutes), and the concentration of each primer is less than 20 nM, and (vii) any combination thereof. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have the following properties: (i) ΔG values for possible combinations of that primer with other primers in the library are all equal to or greater than -10 kcal/mol and (ii) the range of melting temperature of the primers is between 1 to 15 °C. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have the following properties: (i) the length of the target amplicons is between 50 and 100 nucleotides, and (ii) the concentration of each primer is less than 20 nM. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have the following properties: (i) the length of the target amplicons is between 50 and 100 nucleotides and (ii) the length of the annealing step is greater than 5 minutes (such as greater than 10 minutes). In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have the following properties: (i) the length of the target amplicons is between 50 and 100 nucleotides, (ii) the length of the annealing step is greater than 5 minutes (such greater than 10 minutes), and (iii) the concentration of each primer is less than 20 nM, and (vii) any combination thereof.

[0289] In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have one or more of the following properties: (i) the annealing temperature is at least 5 °C (such as at least 6, 8, or 10 °C) greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers; (ii) the annealing temperature is between 5 and 15 °C, inclusive greater than the melting temperature of the primers; (iii) the annealing temperature is at least 5 °C (such as at least 6, 8, or 10 °C) greater than the highest melting temperature of the primers; (iv) the annealing temperature is at least 5 °C (such as at least 6, 8,

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or 10 °C) greater than the average melting temperature of the primers, (v) the annealing temperature is between 4 and 15 °C inclusive greater than the average melting temperature of the primers; and (vi) any combination thereof. In various embodiments, at least 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the primers in the library have one or more of the following properties: (i) the annealing temperature is at least 5 °C (such as at least 6, 8, or 10 °C) greater than the melting temperature (such as the empirically measured or calculated T_m) of the primers and the length of the annealing step is greater than 5 minutes (such greater than 10 minutes); (ii) the annealing temperature is between 5 and 15 °C, inclusive greater than the melting temperature of the primers and the length of the annealing step is greater than 5 minutes (such greater than 10 minutes); (iii) the annealing temperature is at least 5 °C (such as at least 6, 8, or 10 °C) greater than the highest melting temperature of the primers and the length of the annealing step is greater than 5 minutes (such greater than 10 minutes); (iv) the annealing temperature is at least 5 °C (such as at least 6, 8, or 10 °C) greater than the average melting temperature of the primers and the length of the annealing step is greater than 5 minutes (such greater than 10 minutes), (v) the annealing temperature is between 4 and 15 °C inclusive greater than the average melting temperature of the primers and the length of the annealing step is greater than 5 minutes (such greater than 10 minutes); and (vii) any combination thereof. In some embodiments, the guanine-cytosine (GC) content of the primers is between 30% and 80%, inclusive; the range of melting temperatures of the primers is less than 5 °C; and the length of the primers is between 15 to 75 nucleotides, inclusive;

[0290] In some embodiments, some (such as at least 80, 90, or 95%) or all of the adaptors or primers include one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramide, phosphorothioate, and phosphorodithioate linkages. In some embodiments, some (such as at least 80, 90, or 95%) or all of the adaptors or primers include a thiophosphate (such as a monothiophosphate) between the last 3' nucleotide and the second to last 3' nucleotide. In some embodiments, some (such as at least 80, 90, or 95%) or all of the adaptors or primers include a thiophosphate (such as a monothiophosphate) between the last 2, 3, 4, or 5 nucleotides at the 3' end. In some embodiments, some (such as at least 80, 90, or 95%) or all of the adaptors or primers include a thiophosphate (such as a monothiophosphate) between at least 1, 2, 3, 4, or 5 nucleotides out of the last 10 nucleotides at the 3' end. In some embodiments, such primers are less likely to be cleaved or degraded, such primers may be desirable if a polymerase with proof-

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reading ability is used (to reduce or prevent the polymerase from removing nucleotides from the primers). In some embodiments, any of the embodiments involving primers with at least one linkage other than a naturally-occurring phosphodiester linkage are used with a polymerase having proof-reader activity. In some embodiments, the primers do not contain an enzyme cleavage site (such as a protease cleavage site). In some embodiments, equal to or greater than 1, 10, 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 primers in the library are non-naturally occurring nucleic acids (such nucleic acids with one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage).

[0291] In some embodiments, the primers have any combination of two or more of the aspects or embodiments disclosed herein.

[0292] These primer libraries can be used in any of the methods of the invention.

[0293] Exemplary Primers

[0294] The primer design methods of the invention have been used to generate several exemplary primer libraries to human target loci. For example, the primer design methods of the invention were used to generate primer libraries. Each of these libraries is composed of three primers per target locus for 1,200; 2,686; or 10,984 different target loci, respectively. The methods of the invention can also be used to generate libraries to non-human target loci.

[0295] For an experiment using the 2,686-plex library for multiplex PCR followed by sequencing, the percent of the amplified products that were primer dimers was 11.13%, the median depth of read per target that was amplified was 799.5x coverage, the percent of amplified products that were target amplicons out of the amplified products that were not primer dimers was 93.15% (this is the percent of on target reads when reads for amplified primer dimers are ignored); the number of target loci that were not amplified (failed assay count) was 246; the percent of target loci that were not amplified (failed assay percentage) was 9.16%; the percent of target loci that were amplified was 90.84%; and the total number of reads was 2,522,742. For this primer library, the ΔG values for each possible combination of two primers (each possible primer dimer) in the library are all equal to or greater than -3.86 kcal/mol. This -3.86 kcal/mol value was used as a threshold value to select candidate primers that all had a value equal to or greater than (more desirable than) this value from an initial library of candidate primers.

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[0296] For an experiment using the 10,984-plex library for multiplex PCR followed by sequencing, the percent of the amplified products that were primer dimers was 5.50%, the median depth of read per target that was amplified was 1,286.5x coverage, the percent of amplified products that were target amplicons out of the amplified products that were not primer dimers was 60.16% (this is the percent of on target reads when reads for amplified primer dimers were ignored); the number of target loci that were not amplified (failed assay count) was 3,712; the percent of target loci that were not amplified (failed assay percentage) was 33.79%; the percent of target loci that were amplified was 66.21%; and the total number of reads was 25,372,858.

[0297] For an experiment using the 1,200-plex library for multiplex PCR of a sample of only a single cell followed by sequencing, the percent of the amplified products that were primer dimers was 24.13%. This library has primers to human target loci on chromosome 1, chromosome 21, and the X chromosome. For chromosomes 1 and 21, the median depth of read per target that was amplified was 436x coverage; the percent of target loci that were not amplified (failed assay percentage) was 32.69%; and the percent of target loci that were amplified was 67.31%. The total number of reads was 808,106.

[0298] The primer design methods of the invention were also used to generate a library for ~11,000 different target loci (such as amplifying 10,732 different target human loci using 10,732 different primer pairs). For an experiment using this library for multiplex PCR followed by sequencing, the percent of the amplified products that were primer dimers was 14.75%, the median depth of read per target that was amplified was 72.27x coverage, the percent of the amplified products that were target amplicons was 84.32%; the number of target loci that were not amplified (failed assay count) was 118; the percent of target loci that were not amplified (failed assay percentage) was 1.10%; the percent of target loci that were amplified was 98.9%; and the total number of reads was 6,345,782. For this primer library, the ΔG values for each possible combination of two primers (each possible primer dimer) in the library are all equal to or greater than -4.28 kcal/mol. This -4.28 kcal/mol value was used as a threshold value to select candidate primer that all had a value equal to or greater than (more desirable than) this value from an initial library of candidate primers. For the initial candidate primers that were used to select primers for this library, the following interaction cost histogram shows the number of candidate primers for each of the following ranges of ΔG values. This illustrates how the values for the candidate primers compares to the -4.28 kcal/mol threshold value for the final library.

0 to -0.497 kcal/mol : 88357
-0.497 to -0.993 kcal/mol : 30529
-0.993 to -1.49 kcal/mol : 7862
-1.49 to -1.99 kcal/mol : 2639
-1.99 to -2.48 kcal/mol : 1086
-2.48 to -2.98 kcal/mol : 393
-2.98 to -3.48 kcal/mol : 148
-3.48 to -3.97 kcal/mol : 58
-3.97 to -4.47 kcal/mol : 18
-4.47 to -4.97 kcal/mol : 4
-4.97 to -5.46 kcal/mol : 3
-5.46 to -5.96 kcal/mol : 0
-5.96 to -6.46 kcal/mol : 2
-6.46 to -6.95 kcal/mol : 3

[0299] The primer design methods of the invention were also used to generate a library for ~14,000 different target loci (such as amplify 13,392 different target human loci with 13,392 different primer pairs). For an experiment using this library for multiplex PCR followed by sequencing, the percent of the amplified products that were primer dimers was 0.56%, the median depth of read per target that was amplified was 69.09x coverage, the percent of the amplified products that were target amplicons was 99.42%; the number of target loci that were not amplified (failed assay count) was 44; the percent of target loci that were not amplified (failed assay percentage) was 0.33%; the percent of target loci that were amplified was 99.67%; and the total number of reads was 7,772,454.

[0300] The primer design methods of the invention were also used to generate a library composed of three primers per target locus for 19,488 different target loci. Examples 15, 18, and 19 describe the use of this library. During the PCR amplification and sequencing of a genomic sample, 99.4-99.7% of the sequencing reads mapped to the genome, of those, 99.99% of the reads mapped to target loci. For plasma samples with 10 million sequencing reads, typically at least 19,350 of the 19,488 target loci (99.3 %) were amplified and sequenced. For another experiment, the percent of the amplified products that were primer dimers was 1.62%, the median depth of read per target that was amplified was 30x coverage; the percent of the amplified products that were target amplicons was 98.15%; the number of target loci that were not amplified (failed assay count) was 736; the percent of target loci that were not amplified (failed assay percentage) was 0.56%; the percent of target loci that were amplified was 99.44%; and the total number of reads was 6,476,975. For this 19,488-plex library, FIG. 34 is a table of

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the percentage of reads that map to target loci for genomic DNA samples and for samples of a single cell from a cell line for both mother and child samples using this primer library. There was variability in the single cell data which may have resulted from some dead cells being selected, which may have had most of the DNA leaked out. FIG. 35 is an overlay of depth of read for genomic and a single cell sample for different SNPs. FIG. 36 is a table of the percentage of reads that map to target loci for blastoceol fluid and for a single blastocyst cell. The blastoceol fluid produced no mapped reads, possibly due to no DNA being detected. For a single blastocyst, 50-80% of the reads mapped to target loci. FIG. 37 is a graph of reference counts (counts of one allele, such as the "A" allele) divided by total counts for that locus for a single blastocyst cell. For this primer library, the ΔG values for each possible combination of two primers (each possible primer dimer) in the library are all equal to or greater than -3.86 kcal/mol. This -3.86 kcal/mol value was used as a threshold value to select candidate primer that all had a value equal to or greater than (more desirable than) this value from an initial library of candidate primers.

[0301] The primer design methods of the invention were used to generate a library for ~28,000 different target loci (such as amplifying 27,744 different loci with 27,744 different primer pairs). For multiplex PCR and sequencing of genomic DNA samples, 99% of the sequencing reads mapped to target loci. The number of different target human loci that were amplified was 23,776.

[0302] For an experiment using this library, the percent of the amplified products that were primer dimers was 0.63%, the median depth of read per target that was amplified was 20x coverage, the percent of the amplified products that were target amplicons was 99.33%; the number of target loci that were not amplified (failed assay count) was 3,968; the percent of target loci that were not amplified (failed assay percentage) was 14.29%; the percent of target loci that were amplified was 85.71%; and the total number of reads was 4,456,636. For a single cell from a cell line, between 2 and 8% of the reads mapped to target loci.

[0303] The primer design methods of the invention were used to generate a library for ~9,600 different target loci. As described in Example 10, 7.6 million (97%) of reads mapped to the genome, and 6.3 million (80%) of the reads mapped to the targeted SNPs. The average depth of read was 751, and the median depth of read was 396. As described in Example 9, another experiment produced 3.7 million reads mapping to the genome (94%), and of those, 2.9 million

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reads (74%) mapped to targeted SNPs with an average depth of read of 344 and a median depth of read of 255.

[0304] The primer design methods of the invention were used to generate a library for ~2,400 different target loci. As described in Example 12, when four portions were each amplified with ~2,400 primers, 4.5 million reads mapped to targeted SNPs, the average depth of read was 535 and the median depth of read was 412.

[0305] If desired, any of the results may be improved by increasing the number of cells or the amount of nucleic acid template used for the analysis or by optimizing the conditions. For example, if results from single cell samples are not as good as desired for a particular application, a sample with more cells or more nucleic acids may be used instead (such as to decrease the percentage of primer dimers, increase the percentage of target amplicons, or increase the percentage of target loci that are amplified). Samples with more nucleic acids have more template molecules for the primers to bind (instead of primers binding each other and forming primer dimers).

[0306] These primer libraries or primer pools can be used in any of the PCR methods of the invention. In some embodiments, primers from any of the primer pools are used in combination with a universal primer to amplify the target loci. In some embodiments, multiple rounds of PCR are performed in which each round of PCR uses primers from one of the primer pools and a universal primer. In some embodiments, primers from two of the primer pools are used to amplify the target loci. In some embodiments, multiple rounds of PCR are performed. In some embodiments, primers from pools C and B are used for the first round of PCR and then primers from pools A and C are used for the second of PCR. In some embodiments, primers from pools C and B are used for the first round of PCR and then primers from pools A and B are used for the second of PCR.

[0307] In some embodiments, a region that is not specific for a target locus (such as a tag, bar code, or universal binding site) is added to one or more primers of the invention. In various embodiments, the nonspecific region is added to the 5' end of the primer, to the 3' end of the primer, or to an internal region of the primer. In some embodiments, the primers are fragments (such as fragments of at least 10, 20, 30, 40, 50 or more contiguous nucleotides that are less than full-length).

[0308] In some embodiments, the invention provides a library of primers that includes at least 10; 20; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 20,000; 25,000;

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30,000; 40,000; or 50,000 different primers. In some embodiments, the invention provides a library of primers that includes at least 10; 20; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 20,000; 25,000; 30,000; 40,000; or 50,000 different primers.

[0309] Percent identity in reference to nucleic acid sequences refers to the degree of sequence identity between nucleic acid sequences. Percent identity can be determined in various ways that are within the skill in the art, for instance, using publicly available computer software with the default parameters such as Smith Waterman Alignment (Smith and Waterman J. Mol. Biol. 147:195-7, 1981); "BestFit" (Smith and Waterman, Advances in Applied Mathematics, 482-489, 1981); Basic Local Alignment Search Tool (BLAST, Altschul, S. F., W. Gish, et al., J. Mol. Biol. 215: 403-410, 1990; available through the U.S. government's National Center for Biotechnology Information web site at the world wide web at ncbi.nlm.nih.gov), BLAST-2, BLAST-N, WU-BLAST, WU-BLAST-2, ENTREZ (available through the National Center for Biotechnology Information), CLUSTALW, CLUSTAL Omega, or Megalign (DNASTAR, Inc. Madison, Wis.) software. In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared. In general, the length of comparison will generally be at least 20, 30, 40, 45, 50, or more nucleotides.

[0310] In some embodiments, percent identity is calculated by determining the number of matched positions in aligned nucleic acid sequences, dividing the number of matched positions by the total number of aligned nucleotides, and multiplying by 100. A matched position refers to a position in which identical nucleotides occur at the same position in aligned nucleic acid sequences. The percent identity over a particular length is determined by counting the number of matched positions over that length and dividing that number by the length followed by multiplying the resulting value by 100. For example, if (i) a 500-nucleotide nucleic acid target sequence is compared to a subject nucleic acid sequence, (ii) an alignment program presents 200 nucleotides from the target sequence aligned with a region of the subject sequence where the first and last nucleotides of that 200-nucleotide region are matches, and (iii) the number of matches over those 200 aligned nucleotides is 180, then the 500-nucleotide nucleic acid target sequence contains a length of 200 and a sequence identity over that length of 90% (*i.e.*, 180, 200x100=90).

[0311] Hybridization conditions resulting in a particular degree of stringency will vary depending upon the nature of the hybridization method and the composition and length of the

hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (such as the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions for attaining particular degrees of stringency are discussed in Sambrook *et al.,* (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, N.Y. (chapters 9 and 11); Nucleic Acid Hybridization, A Practical Approach, Ed. Hames, B. D. and Higgins, S. J., IRL Press, 1985; Ausubel *et al.* Current Protocols in Molecular Biology, Wiley, New York 1994; and U.S Pat. No. 8,357,488, filed May 16, 2008. In some embodiments, very high stringency hybridization conditions includes an overnight incubation at 42° C in a solution comprising 50% formamide, 5xSSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5xDenhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1xSSC at about 65° C. The following is an exemplary set of hybridization conditions and is not limiting:

Very High Stringency (Detects Sequences that Share at Least 90% Identity)

Hybridization: 5xSSC at 65° C for 16 hours

Wash twice: 2xSSC at room temperature for 15 minutes each

Wash twice: 0.5xSSC at 65° C for 20 minutes each

High Stringency (Detects Sequences that Share at Least 80% Identity)

Hybridization: 5x-6xSSC at 65° C-70° C. for 16-20 hours

Wash twice: 2xSSC at room temperature for 5-20 minutes each

Wash twice: 1xSSC at 55° C-70° C for 30 minutes each

Low Stringency (Detects Sequences that Share at Least 50% Identity)

Hybridization: 6xSSC at room temperature to 55° C. for 16-20 hours

Wash at least twice: 2x-3xSSC at room temperature to 55° C for 20-30 minutes each

[0312] These primers can be used in any of the primer libraries or methods of the invention.

Exemplary Primer Libraries for Detection of Recombination

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[0313] In some embodiments, primers in the primer library are designed to determine whether or not recombination occurred at one or more known recombination hotspots (such as crossovers between homologous human chromosomes). Knowing what crossovers occurred between chromosomes allows more accurate phased genetic data to be determined for an individual. Recombination hotspots are local regions of chromosomes in which recombination events tend to be concentrated. Often they are flanked by "coldspots," regions of lower than average frequency of recombination. Recombination hotspots tend to share a similar morphology and are approximately 1 to 2 kb in length. The hotspot distribution is positively correlated with GC content and repetitive element distribution. A partially degenerated 13-mer motif CCNCCNTNNCCNC plays a role in some hotspot activity. It has been shown that the zinc finger protein called PRDM9 binds to this motif and initiates recombination at its location. The average distance between the centers of recombination hot spots is reported to be ~80 kb. In some embodiments, the distance between the centers of recombination hot spots ranges between ~3 kb to ~100 kb. Public databases include a large number of known human recombination hotspots, such as the HUMHOT and International HapMap Project databases (see, for example, Nishant *et al.,* "HUMHOT: a database of human meiotic recombination hot spots," Nucleic Acids Research, 34: D25–D28, 2006, Database issue; Mackiewicz *et al.,* "Distribution of Recombination Hotspots in the Human Genome – A Comparison of Computer Simulations with Real Data" PLoS ONE 8(6): e65272, doi:10.1371/journal.pone.0065272; and the world wide web at hapmap.ncbi.nlm.nih.gov/downloads/index.html.en, which are each hereby incorporated by reference in its entirety).

[0314] In some embodiments, primers in the primer library are clustered at or near recombination hotspots (such as known human recombination hotspots). In some embodiments, the corresponding amplicons are used to determine the sequence within or near a recombination hotspot to determine whether or not recombination occurred at that particular hotspot (such as whether the sequence of the amplicon is the sequence expected if a recombination had occurred or the sequence expected if a recombination had not occurred). In some embodiments, primers are designed to amplify part or all of a recombination hotspot (and optionally sequence flanking a recombination hotspot). In some embodiments, long read sequencing (such as sequencing using the Moleculo Technology developed by Illumina to sequence up to ~10 kb) or paired end sequencing is used to sequence part or all of a recombination hotspot. Knowledge of whether or not a recombination event occurred can be used to determine which haplotype blocks flank

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the hotspot. If desired, the presence of particular haplotype blocks can be confirmed using primers specific to regions within the haplotype blocks. In some embodiments, it is assumed there are no crossovers between known recombination hotspots. In some embodiments, primers in the primer library are clustered at or near the ends of chromosomes. For example, such primers can be used to determine whether or not a particular arm or section at the end of a chromosome is present. In some embodiments, primers in the primer library are clustered at or near recombination hotspots and at or near the ends of chromosomes.

[0315] In some embodiments, the primer library includes one or more primers (such as at least 5; 10; 50; 100; 200; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 20,000; 25,000; 30,000; 40,000; or 50,000 different primers or different primer pairs) that are specific for a recombination hotspot (such as a known human recombination hotspot) and/or are specific for a region near a recombination hotspot (such as within 10, 8, 5, 3, 2, 1, or 0.5 kb of the 5' or 3' end of a recombination hotspot). In some embodiments, at least 1, 5, 10, 20, 40, 60, 80, 100, or 150 different primer (or primer pairs) are specific for the same recombination hotspot, or are specific for the same recombination hotspot or a region near the recombination hotspot. In some embodiments, at least 1, 5, 10, 20, 40, 60, 80, 100, or 150 different primer (or primer pairs) are specific for a region between recombination hotspots (such as a region unlikely to have undergone recombination); these primers can be used to confirm the presence of haplotype blocks (such as those that would be expected depending on whether or not recombination has occurred). In some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the primers in the primer library are specific for a recombination hotspot and/or are specific for a region near a recombination hotspot (such as within 10, 8, 5, 3, 2, 1, or 0.5 kb of the 5' or 3' end of the recombination hotspot). In some embodiments, the primer library is used to determine whether or not recombination has occurred at greater than or equal to 5; 10; 50; 100; 200; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 20,000; 25,000; 30,000; 40,000; or 50,000 different recombination hotspots (such as known human recombination hotspots). In some embodiments, the regions targeted by primers to a recombination hotspot or nearby region are approximately evenly spread out along that portion of the genome. In some embodiments, at least 1, 5, 10, 20, 40, 60, 80, 100, or 150 different primer (or primer pairs) are specific for the a region at or near the end of a chromosome (such as a region within 20, 10, 5, 1, 0.5, 0.1, 0.01, or 0.001 mb from the end of a chromosome). In some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the primers in the primer library are specific for the a region at or near the end of a

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chromosome (such as a region within 20, 10, 5, 1, 0.5, 0.1, 0.01, or 0.001 mb from the end of a chromosome). In some embodiments, at least 1, 5, 10, 20, 40, 60, 80, 100, or 150 different primer (or primer pairs) are specific for a region within a potential microdeletion in a chromosome. In some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the primers in the primer library are specific for a region within a potential microdeletion in a chromosome. In some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the primers in the primer library are specific for a recombination hotspot, a region near a recombination hotspot, a region at or near the end of a chromosome, or a region within a potential microdeletion in a chromosome. In some embodiments, the primers have one or more of the properties described herein. Other embodiments are disclosed in U.S.S.N. 61/987,407, filed May 1, 2014 and 62/066,514, filed Oct. 21, 2014.

Exemplary Kits

[0316] In one aspect, the invention features a kit, such as a kit for amplifying target loci in a nucleic acid sample for detecting deletions and/or duplications of chromosome segments or entire chromosomes using any of the methods described herein). In some embodiments, the kit can include any of the primer libraries of the invention. In an embodiment, the kit comprises a plurality of inner forward primers and optionally a plurality of inner reverse primers, and optionally outer forward primers and outer reverse primers, where each of the primers is designed to hybridize to the region of DNA immediately upstream and/or downstream from one of the target sites (*e.g.*, polymorphic sites) on the target chromosome(s) or chromosome segment(s), and optionally additional chromosomes or chromosome segments. In some embodiments, the kit includes instructions for using the primer library to amplify the target loci, such as for detecting one or more deletions and/or duplications of one or more chromosome segments or entire chromosomes using any of the methods described herein.

[0317] In certain embodiments, kits of the invention provide primer pairs for detecting chromosomal aneuploidy and CNV determination, such as primer pairs for massively multiplex reactions for detecting chromosomal aneuploidy such as CNV (CoNVERGe) (Copy Number Variant Events Revealed Genotypically) and/or SNVs. In these embodiments, the kits can include between at least 100, 200, 250, 300, 500, 1000, 2000, 2500, 3000, 5000, 10,000, 20,000, 25,000, 28,000, 50,000, or 75,000 and at most 200, 250, 300, 500, 1000, 2000, 2500, 3000, 5000,

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10,000, 20,000, 25,000, 28,000, 50,000, 75,000, or 100,000 primer pairs that are shipped together. The primer pairs can be contained in a single vessel, such as a single tube or box, or multiple tubes or boxes. In certain embodiments, the primer pairs are pre-qualified by a commercial provider and sold together, and in other embodiments, a customer selects custom gene targets and/or primers and a commercial provider makes and ships the primer pool to the customer neither in one tube or a plurality of tubes. In certain exemplary embodiments, the kits include primers for detecting both CNVs and SNVs, especially CNVs and SNVs known to be correlated to at least one type of cancer.

[0318] Kits for circulating DNA detection according to some embodiments of the present invention, include standards and/or controls for circulating DNA detection. For example, in certain embodiments, the standards and/or controls are sold and optionally shipped and packaged together with primers used to perform the amplification reactions provided herein, such as primers for performing CoNVERGe. In certain embodiments, the controls include polynucleotides such as DNA, including isolated genomic DNA that exhibits one or more chromosomal aneuploidies such as CNV and/or includes one or more SNVs. In certain embodiments, the standards and/or controls are called PlasmArt standards and include polynucleotides having sequence identity to regions of the genome known to exhibit CNV, especially in certain inherited diseases, and in certain disease states such as cancer, as well as a size distribution that reflects that of cfDNA fragments naturally found in plasma. Exemplary methods for making PlasmArt standards are provided in the examples herein. In general, genomic DNA from a source known to include a chromosomal aneuoploidy is isolated, fragmented, purified and size selected.

[0319] Accordingly, artificial cfDNA polynucleotide standards and/or controls can be made by spiking isolated polynucleotide samples prepared as summarized above, into DNA samples known not to exhibit a chromosomal aneuploidy and/or SNVs, at concentrations similar to those observed for cfDNA in vivo, such as between, for example, 0.01% and 20%, 0.1 and 15%, or .4 and 10% of DNA in that fluid. These standards/controls can be used as controls for assay design, characterization, development, and/or validation, and as quality control standards during testing, such as cancer testing performed in a CLIA lab and/or as standards included in research use only or diagnostic test kits.

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Exemplary Amplicons

[0320] In one aspect, the invention provides a composition comprising at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical amplicons) in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of at least 100 different target loci (*e.g.*, at least 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical target loci) using at least 100 different primers or primer pairs (*e.g.*, at least 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers or primer pairs) in one reaction volume. In some embodiments, (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the range of the length of the target amplicons is between 5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, the composition includes at least 1,000 different amplicons in solution in one reaction volume; wherein the amplicons are produced from the simultaneous PCR amplification of at least 1,000 different target human loci using at least 1,000 different primers in one reaction volume; wherein (i) less than 20% of the amplicons are primer dimers, and (ii) at least 80% of the amplicons comprise one of the target human loci and are between 50 and 100 nucleotides in length, inclusive. In some embodiments, the composition consists essentially of, or consists of one or more of the following: amplicons, primers (such as any of the primers disclosed herein), free nucleotide(s), non-human or non-naturally occurring enzyme(s), buffer(s), or any combination thereof.

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[0321] In some embodiments, a large percentage or substantially all of the primers used for the multiplex PCR method are consumed during the PCR reaction or are removed from the reaction volume after the PCR amplification. In some embodiments, at least 80, 90, 92, 94, 96, 98, 99, or 100% of the primer molecules are extended to form amplified products. In some embodiments, for at least 80, 90, 92, 94, 96, 98, 99, or 100% of target loci, at least 80, 90, 92, 94, 96, 98, 99, or 100% of the primer molecules to that target loci are extended to form amplified products. In some embodiments, multiple cycles are performed until all or substantially all of the primers are consumed. If desired, a higher percentage of the primers can be consumed by decreasing the initial primer concentration and/or increasing the number of PCR cycles that are performed. In some embodiments, at least 80, 90, 95, 96, 97, 98, 99, or 100% of the nucleic acids in the composition are amplicons (instead of unextended dimers).

[0322] In one aspect, the invention provides a composition comprising at least 100 different primers or primer pairs (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical primers or primer pairs) and at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical amplicons) in solution in one reaction volume. In some embodiments, the amplicons are produced from the simultaneous PCR amplification of at least 100 different target loci (*e.g.*, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical target loci) using the primers or primer pairs in one reaction volume. In some embodiments, (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons. In some embodiments, the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 to 75 nucleotides, inclusive. In some embodiments, the range of the length of the target amplicons is less than 50, 25, 15, 10, or 5 nucleotides. In some embodiments, the range of the length of the target amplicons is between

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5 to 50 nucleotides, such as 5 to 25 nucleotides, 5 to 15 nucleotides, or 5 to 10 nucleotides, inclusive. In some embodiments, the composition comprising at least 1,000 different primers and at least 1,000 different amplicons in solution in one reaction volume; wherein the amplicons are produced from the simultaneous PCR amplification of at least 1,000 different target human loci with the primers in one reaction volume; wherein (i) less than 20% of the amplicons are primer dimers, and (ii) at least 80% of the amplicons comprise one of the target loci and are between 50 and 100 nucleotides in length, inclusive. In some embodiments, the composition consists essentially of, or consists of one or more of the following: amplicons, primers (such as any of the primers disclosed herein), free nucleotide(s), non-human or non-naturally occurring enzyme(s), buffer(s), or any combination thereof.

[0323] In some embodiments, the amplification of different target loci is substantially uniform. In some embodiments, target loci (such as nonpolymorphic target loci or polymorphic target loci that are amplified regardless of what allele is present at the polymorphic site) that were present in the same amount (or substantially the same amount) in the initial unamplified sample are also present in substantially the same amount in the PCR-amplified products. In some embodiments, for at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different targets, the target loci that is amplified the most out of these targets (which can be all of the targets or a subset of the targets for a library) is amplified less than 2,000; 1,500; 1,000; 500, 400, 300, 200, 100%, 50, 20, 10, 5, or 2% more than the target loci that is amplified the least out of these targets. In some embodiments, for at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target amplicons, the target amplicon in greatest abundance out of these target amplicons (which can be all of the target amplicons or a subset of the target amplicons produced by a library) is present in an amount that is less than 2,000; 1,500; 1,000; 500, 400, 300, 200, 100%, 50, 20, 10, 5, or 2% more than the target amplicon in least abundance out of these target amplicons. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target amplicons are present in an amount that is at least 5, 10, 15, 20, 40, 50, 60, 70,. 80, or 90% of the amount of the target amplicon in greatest abundance. In some embodiments, for at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000

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different amplicons that are produced by multiplex PCR and then sequenced, at least 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target amplicons produce a number of sequencing reads within 20, 30, 50, or 80% above or below the mean number of sequences for target amplicons. If desired, the uniformity can be increased by using primers with more similar primer lengths, target amplicon lengths, GC contents, melting temperatures, or any combination thereof. In some embodiments, the uniformity can be increased by using TMAC in the reaction volume during amplification. In some embodiments, having most or all the primers consumed in the PCR reaction increases the uniformity of amplification.

[0324] If desired, the uniformity in DOR can be measured using standard methods such as depth of read slope (DOR slope), normalized median depth of read (nmDOR), or breadth of read (BOR). DOR slope represents the slope of the line in the linear portion of a list of loci sorted in descending DOR order. Closer to zero is better, as it represents a flat line. In some embodiments, the uniformity in DOR can be measured using the Percent of reads in the 90th – 95th Percentile. For this measurement, the loci are sorted in descending DOR order. In the ideal DOR distribution, the 90th-95th percentile should contain 5% of reads. The reads of all loci between the 90th Percentile and 95th percentile are counted and divided by the total reads for all loci. In one experiment, the DOR slope versus percent of reads in the 90th – 95th percentile for all samples had an $R^2 = 0.81$.

[0325] In some embodiments, the magnitude of the DOR slope is less than 0.005, 0.001, 0.0005, 0.0001, 0.00005, 0.00001, 0.000005, or 0.000001. In some embodiments, the magnitude of the DOR slope is between 0 and 0.005, such as 0.000001 to 0.005, such as between 0.000005 to 0.00001, 0.00001 to 0.00005, 0.00005 to 0.0001, 0.0001 to 0.0005, 0.0005 to 0.001, or 0.001 to 0.005, inclusive. In some embodiments, the percent of reads in the 90th – 95th percentile is between 0.2 and 9%, such as between 1 to 8%, 2 to 7%, 0.2 to 1.0%, 1 to 2%, 2 to 3%, 2 to 4%, 3 to 4%, 4 to 5%, 5 to 6%, or 6 to 8%, or 7 to 9& inclusive. In some embodiments, the invention features a composition comprising at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical amplicons) with the magnitude of the DOR slope in any of these ranges or with a percent of reads in the 90th – 95th percentile in any of these ranges. In some embodiments, the amplification method produces at least 100 different amplicons (*e.g.*, at least 300, 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 non-identical

amplicons) with the magnitude of the DOR slope in any of these ranges or with a percent of reads in the 90th – 95th percentile in any of these ranges.

Exemplary Multiplex PCR Methods

[0326] In one aspect, the invention features methods of amplifying target loci in a nucleic acid sample that involve (i) contacting the nucleic acid sample with a library of primers that simultaneously hybridize to least 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture; and (ii) subjecting the reaction mixture to primer extension reaction conditions (such as PCR conditions) to produce amplified products that include target amplicons. In some embodiments, the method also includes determining the presence or absence of at least one target amplicon (such as at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target amplicons). In some embodiments, the method also includes determining the sequence of at least one target amplicon (such as at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target amplicons). In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified. In some embodiments, at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.5, or 100% of the target loci are amplified at least 5, 10, 20, 40, 50, 60, 80, 100, 120, 150, 200, 300, or 400-fold. In various embodiments, less than 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers. In some embodiments, the method involves multiplex PCR and sequencing (such as high throughput sequencing).

[0327] In various embodiments, long annealing times and/or low primer concentrations are used. In various embodiments, the length of the annealing step is greater than 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes. In various embodiments, the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive. In various embodiments, the length of the annealing step is greater than 5 minutes (such greater than 10, or 15 minutes), and the concentration of each primer is less than 20 nM. In various embodiments, the length of the annealing step is greater than 5

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minutes (such greater than 10, or 15 minutes), and the concentration of each primer is between 1 to 20 nM, or 1 to 10 nM, inclusive. In various embodiments, the length of the annealing step is greater than 20 minutes (such as greater than 30, 45, 60, or 90 minutes), and the concentration of each primer is less than 1 nM.

[0328] At high level of multiplexing, the solution may become viscous due to the large amount of primers in solution. If the solution is too viscous, one can reduce the primer concentration to an amount that is still sufficient for the primers to bind the template DNA. In various embodiments, less than 60,000 different primers are used and the concentration of each primer is less than 20 nM, such as less than 10 nM or between 1 and 10 nM, inclusive. In various embodiments, more than 60,000 different primers (such as between 60,000 and 120,000 different primers) are used and the concentration of each primer is less than 10 nM, such as less than 5 nM or between 1 and 10 nM, inclusive.

[0329] It was discovered that the annealing temperature can optionally be higher than the melting temperatures of some or all of the primers (in contrast to other methods that use an annealing temperature below the melting temperatures of the primers) (Example 25). The melting temperature (T_m) is the temperature at which one-half (50%) of a DNA duplex of an oligonucleotide (such as a primer) and its perfect complement dissociates and becomes single strand DNA. The annealing temperature (T_A) is the temperature one runs the PCR protocol at. For prior methods, it is usually 5 C below the lowest T_m of the primers used, thus close to all possible duplexes are formed (such that essentially all the primer molecules bind the template nucleic acid). While this is highly efficient, at lower temperatures there are more unspecific reactions bound to occur. One consequence of having too low a T_A is that primers may anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated. In some embodiments of the present inventions, the T_A is higher than (T_m), where at a given moment only a small fraction of the targets have a primer annealed (such as only ~1-5%). If these get extended, they are removed from the equilibrium of annealing and dissociating primers and target (as extension increases T_m quickly to above 70 C), and a new ~1-5% of targets has primers. Thus, by giving the reaction long time for annealing, one can get ~100% of the targets copied per cycle. Thus, the most stable molecule pairs (those with perfect DNA pairing between the primer and the template DNA) are preferentially extended to produce the correct target amplicons. For example, the same experiment was performed with 57°C as the annealing temperature and with 63°C as the annealing temperature with primers that had a

melting temperature below 63°C. When the annealing temperature was 57°C, the percent of mapped reads for the amplified PCR products was as low as 50% (with ~ 50% of the amplified products being primer-dimer). When the annealing temperature was 63°C, the percentage of amplified products that were primer dimer dropped to ~2%.

[0330] In various embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers, and the length of the annealing step (per PCR cycle) is greater than 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes.

[0331] In various embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In various embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers, and the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive.

[0332] In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers, and the length of the

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annealing step (per PCR cycle) is greater than 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes

[0333] In some embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers. In some embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the highest melting temperature (such as the empirically measured or calculated T_m) of the primers, and the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive.

[0334] In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In some embodiments, the annealing temperature is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 15 °C greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers, and the length of the annealing step (per PCR cycle) is greater than 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes.

[0335] In some embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or all of the non-identical primers. In some embodiments, the annealing temperature is between 1 and 15 °C (such as between 1 to 10, 1 to 5, 1 to 3, 3 to 5, 5 to 10, 5 to 8, 8 to 10, 10 to 12, or 12 to 15 °C, inclusive) greater than the average melting temperature (such as the empirically measured or calculated T_m) of at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; 100,000; or

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all of the non-identical primers, and the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive.

[0336] In some embodiments, the annealing temperature is between 50 to 70°C, such as between 55 to 60, 60 to 65, or 65 to 70°C, inclusive. In some embodiments, the annealing temperature is between 50 to 70°C, such as between 55 to 60, 60 to 65, or 65 to 70°C, inclusive, and either (i) the length of the annealing step (per PCR cycle) is greater than 3, 5, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, or 180 minutes or (ii) the length of the annealing step (per PCR cycle) is between 5 and 180 minutes, such as 5 to 60, 10 to 60, 5 to 30, or 10 to 30 minutes, inclusive.

[0337] In some embodiments, one or more of the following conditions are used for empirical measurement of T_m or are assumed for calculation of T_m: temperature: of 60.0 °C, primer concentration of 100 nM, and/or salt concentration of 100 mM. In some embodiments, other conditions are used, such as the conditions that will be used for multiplex PCR with the library. In some embodiments, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 7.5 nM of each primer, and 50 mM TMAC, at pH 8.1 is used. In some embodiments, the T_m is calculated using the Primer3 program (libprimer3 release 2.2.3) using the built-in SantaLucia parameters (the world wide web at primer3.sourceforge.net, which is hereby incorporated by reference in its entirety). For example, the T_m values may be calculated using the method in Example 25. In some embodiments, the calculated melting temperature for a primer is the temperature at which half of the primers molecules are expected to be annealed. As discussed above, even at a temperature higher than the calculated melting temperature, a percentage of primers will be annealed, and therefore PCR extension is possible. In some embodiments, the empirically measured Tm (the actual Tm) is determined by using a thermostatted cell in a UV spectrophotometer. In some embodiments, temperature is plotted vs. absorbance, generating an S-shaped curve with two plateaus. The absorbance reading halfway between the plateaus corresponds to Tm.

[0338] In some embodiments, the absorbance at 260 nm is measured as a function of temperature on an ultrospec 2100 pr UV/visible spectrophotometer (Amershambiosciences) (see, *e.g.*, Takiya *et al.*, "An empirical approach for thermal stability (Tm) prediction of PNA/DNA duplexes," Nucleic Acids Symp Ser (Oxf); (48):131-2, 2004, which is hereby incorporated by reference in its entirety). In some embodiments, absorbance at 260 nm is measured by decreasing the temperature in steps of 2 °C per minute from 95 to 20 °C. In some embodiments, a primer and its perfect complement (such as 2 uM of each paired oligomer) are mixed and then annealing is performed by heating the sample to 95 °C, keeping it there for 5 minutes, followed by cooling

to room temperature during 30 minutes, and keeping the samples at 95 °C for at least 60 minutes. In some embodiments, melting temperature is determined by analyzing the data using SWIFT Tm software. In some embodiments of any of the methods of the invention, the method includes empirically measuring or calculating (such as calculating with a computer) the melting temperature for at least 50, 80, 90, 92, 94, 96, 98, 99, or 100% of the primers in the library either before or after the primers are used for PCR amplification of target loci.

[0339] In some embodiments, the library comprises a microarray. In some embodiments, the library does not comprise a microarray.

[0340] In some embodiments, most or all of the primers are extended to form amplified products. Having all the primers consumed in the PCR reaction increases the uniformity of amplification of the different target loci since the same or similar number of primer molecules are converted to target amplicons for each target loci. In some embodiment, at least 80, 90, 92, 94, 96, 98, 99, or 100% of the primer molecules are extended to form amplified products. In some embodiments, for at least 80, 90, 92, 94, 96, 98, 99, or 100% of target loci, at least 80, 90, 92, 94, 96, 98, 99, or 100% of the primer molecules to that target loci are extended to form amplified products. In some embodiments, multiple cycles are performed until this percentage of the primers are consumed. In some embodiments, multiple cycles are performed until all or substantially all of the primers are consumed. If desired, a higher percentage of the primers can be consumed by decreasing the initial primer concentration and/or increasing the number of PCR cycles that are performed.

[0341] In some embodiments, the PCR methods may be performed with microliter reaction volumes, for which it can be harder to achieve specific PCR amplification (due to the lower local concentration of the template nucleic acids) compared to nanoliter or picoliter reaction volumes used in microfluidics applications. In some embodiments, the reaction volume is between 1 and 60 uL, such as between 5 and 50 uL, 10 and 50 uL, 10 and 20 uL, 20 and 30 uL, 30 and 40 uL, or 40 to 50 uL, inclusive.

[0342] In an embodiment, a method disclosed herein uses highly efficient highly multiplexed targeted PCR to amplify DNA followed by high throughput sequencing to determine the allele frequencies at each target locus. The ability to multiplex more than about 50 or 100 PCR primers in one reaction volume in a way that most of the resulting sequence reads map to targeted loci is novel and non-obvious. One technique that allows highly multiplexed targeted PCR to perform in a highly efficient manner involves designing primers that are unlikely to hybridize with one

another. The PCR probes, typically referred to as primers, are selected by creating a thermodynamic model of potentially adverse interactions between at least 300; at least 500; at least 750; at least 1,000; at least 2,000; at least 5,000; at least 7,500; at least 10,000; at least 20,000; at least 25,000; at least 30,000; at least 40,000; at least 50,000; at least 75,000; or at least 100,000 potential primer pairs, or unintended interactions between primers and sample DNA, and then using the model to eliminate designs that are incompatible with other the designs in the pool. Another technique that allows highly multiplexed targeted PCR to perform in a highly efficient manner is using a partial or full nesting approach to the targeted PCR. Using one or a combination of these approaches allows multiplexing of at least 300, at least 800, at least 1,200, at least 4,000 or at least 10,000 primers in a single pool with the resulting amplified DNA comprising a majority of DNA molecules that, when sequenced, will map to targeted loci. Using one or a combination of these approaches allows multiplexing of a large number of primers in a single pool with the resulting amplified DNA comprising greater than 50%, greater than 60%, greater than 67%, greater than 80%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or greater than 99.5% DNA molecules that map to targeted loci.

[0343] In some embodiments the detection of the target genetic material may be done in a multiplexed fashion. The number of genetic target sequences that may be run in parallel can range from one to ten, ten to one hundred, one hundred to one thousand, one thousand to ten thousand, ten thousand to one hundred thousand, one hundred thousand to one million, or one million to ten million. Prior attempts to multiplex more than 100 primers per pool have resulted in significant problems with unwanted side reactions such as primer-dimer formation.

Targeted PCR

[0344] In some embodiments, PCR can be used to target specific locations of the genome. In plasma samples, the original DNA is highly fragmented (typically less than 500 bp, with an average length less than 200 bp). In PCR, both forward and reverse primers anneal to the same fragment to enable amplification. Therefore, if the fragments are short, the PCR assays must amplify relatively short regions as well. Like MIPS, if the polymorphic positions are too close the polymerase binding site, it could result in biases in the amplification from different alleles. Currently, PCR primers that target polymorphic regions, such as those containing SNPs, are

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typically designed such that the 3' end of the primer will hybridize to the base immediately adjacent to the polymorphic base or bases. In an embodiment of the present disclosure, the 3' ends of both the forward and reverse PCR primers are designed to hybridize to bases that are one or a few positions away from the variant positions (polymorphic sites) of the targeted allele. The number of bases between the polymorphic site (SNP or otherwise) and the base to which the 3' end of the primer is designed to hybridize may be one base, it may be two bases, it may be three bases, it may be four bases, it may be five bases, it may be six bases, it may be seven to ten bases, it may be eleven to fifteen bases, or it may be sixteen to twenty bases. The forward and reverse primers may be designed to hybridize a different number of bases away from the polymorphic site.

[0345] PCR assay can be generated in large numbers, however, the interactions between different PCR assays makes it difficult to multiplex them beyond about one hundred assays. Various complex molecular approaches can be used to increase the level of multiplexing, but it may still be limited to fewer than 100, perhaps 200, or possibly 500 assays per reaction. Samples with large quantities of DNA can be split among multiple sub-reactions and then recombined before sequencing. For samples where either the overall sample or some subpopulation of DNA molecules is limited, splitting the sample would introduce statistical noise. In an embodiment, a small or limited quantity of DNA may refer to an amount below 10 pg, between 10 and 100 pg, between 100 pg and 1 ng, between 1 and 10 ng, or between 10 and 100 ng. Note that while this method is particularly useful on small amounts of DNA where other methods that involve splitting into multiple pools can cause significant problems related to introduced stochastic noise, this method still provides the benefit of minimizing bias when it is run on samples of any quantity of DNA. In these situations a universal pre-amplification step may be used to increase the overall sample quantity. Ideally, this pre-amplification step should not appreciably alter the allelic distributions.

[0346] In an embodiment, a method of the present disclosure can generate PCR products that are specific to a large number of targeted loci, specifically 1,000 to 5,000 loci, 5,000 to 10,000 loci or more than 10,000 loci, for genotyping by sequencing or some other genotyping method, from limited samples such as single cells or DNA from body fluids. Currently, performing multiplex PCR reactions of more than 5 to 10 targets presents a major challenge and is often hindered by primer side products, such as primer dimers, and other artifacts. When detecting target sequences using microarrays with hybridization probes, primer dimers and other artifacts

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may be ignored, as these are not detected. However, when using sequencing as a method of detection, the vast majority of the sequencing reads would sequence such artifacts and not the desired target sequences in a sample. Methods described in the prior art used to multiplex more than 50 or 100 reactions in one reaction volume followed by sequencing will typically result in more than 20%, and often more than 50%, in many cases more than 80% and in some cases more than 90% off-target sequence reads.

[0347] In general, to perform targeted sequencing of multiple (n) targets of a sample (greater than 50, greater than 100, greater than 500, or greater than 1,000), one can split the sample into a number of parallel reactions that amplify one individual target. This has been performed in PCR multiwell plates or can be done in commercial platforms such as the FLUIDIGM ACCESS ARRAY (48 reactions per sample in microfluidic chips) or DROPLET PCR by RAIN DANCE TECHNOLOGY (100s to a few thousands of targets). Unfortunately, these split-and-pool methods are problematic for samples with a limited amount of DNA, as there is often not enough copies of the genome to ensure that there is one copy of each region of the genome in each well. This is an especially severe problem when polymorphic loci are targeted, and the relative proportions of the alleles at the polymorphic loci are needed, as the stochastic noise introduced by the splitting and pooling will cause very poorly accurate measurements of the proportions of the alleles that were present in the original sample of DNA. Described here is a method to effectively and efficiently amplify many PCR reactions that is applicable to cases where only a limited amount of DNA is available. In an embodiment, the method may be applied for analysis of single cells, body fluids, mixtures of DNA such as the free floating DNA found in maternal plasma, biopsies, environmental and/or forensic samples.

[0348] In an embodiment, the targeted sequencing may involve one, a plurality, or all of the following steps. a) Generate and amplify a library with adaptor sequences on both ends of DNA fragments. b) Divide into multiple reactions after library amplification. c) Generate and optionally amplify a library with adaptor sequences on both ends of DNA fragments. d) Perform 1000- to 10,000-plex amplification of selected targets using one target specific "Forward" primer per target and one tag specific primer. e) Perform a second amplification from this product using "Reverse" target specific primers and one (or more) primer specific to a universal tag that was introduced as part of the target specific forward primers in the first round. f) Perform a 1000-plex preamplification of selected target for a limited number of cycles. g) Divide the product into multiple aliquots and amplify subpools of targets in individual reactions (for example, 50

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to 500-plex, though this can be used all the way down to singleplex. h) Pool products of parallel subpools reactions. i) During these amplifications primers may carry sequencing compatible tags (partial or full length) such that the products can be sequenced.

Highly Multiplexed PCR

[0349] Disclosed herein are methods that permit the targeted amplification of over a hundred to tens of thousands of target sequences (*e.g.,* SNP loci) from a nucleic acid sample such as genomic DNA obtained from plasma. The amplified sample may be relatively free of primer dimer products and have low allelic bias at target loci. If during or after amplification the products are appended with sequencing compatible adaptors, analysis of these products can be performed by sequencing.

[0350] Performing a highly multiplexed PCR amplification using methods known in the art results in the generation of primer dimer products that are in excess of the desired amplification products and not suitable for sequencing. These can be reduced empirically by eliminating primers that form these products, or by performing *in silico* selection of primers. However, the larger the number of assays, the more difficult this problem becomes.

[0351] One solution is to split the 5000-plex reaction into several lower-plexed amplifications, *e.g.* one hundred 50-plex or fifty 100-plex reactions, or to use microfluidics or even to split the sample into individual PCR reactions. However, if the sample DNA is limited, such as in non-invasive prenatal diagnostics from pregnancy plasma, dividing the sample between multiple reactions should be avoided as this will result in bottlenecking.

[0352] Described herein are methods to first globally amplify the plasma DNA of a sample and then divide the sample up into multiple multiplexed target enrichment reactions with more moderate numbers of target sequences per reaction. In an embodiment, a method of the present disclosure can be used for preferentially enriching a DNA mixture at a plurality of loci, the method comprising one or more of the following steps: generating and amplifying a library from a mixture of DNA where the molecules in the library have adaptor sequences ligated on both ends of the DNA fragments, dividing the amplified library into multiple reactions, performing a first round of multiplex amplification of selected targets using one target specific "forward" primer per target and one or a plurality of adaptor specific universal "reverse" primers. In an embodiment, a method of the present disclosure further includes performing a second

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amplification using "reverse" target specific primers and one or a plurality of primers specific to a universal tag that was introduced as part of the target specific forward primers in the first round. In an embodiment, the method may involve a fully nested, hemi-nested, semi-nested, one sided fully nested, one sided hemi-nested, or one sided semi-nested PCR approach. In an embodiment, a method of the present disclosure is used for preferentially enriching a DNA mixture at a plurality of loci, the method comprising performing a multiplex preamplification of selected targets for a limited number of cycles, dividing the product into multiple aliquots and amplifying subpools of targets in individual reactions, and pooling products of parallel subpools reactions. Note that this approach could be used to perform targeted amplification in a manner that would result in low levels of allelic bias for 50-500 loci, for 500 to 5,000 loci, for 5,000 to 50,000 loci, or even for 50,000 to 500,000 loci. In an embodiment, the primers carry partial or full length sequencing compatible tags.

[0353] The workflow may entail (1) extracting DNA such as plasma DNA, (2) preparing fragment library with universal adaptors on both ends of fragments, (3) amplifying the library using universal primers specific to the adaptors, (4) dividing the amplified sample "library" into multiple aliquots, (5) performing multiplex (*e.g.* about 100-plex, 1,000, or 10,000-plex with one target specific primer per target and a tag-specific primer) amplifications on aliquots, (6) pooling aliquots of one sample, (7) barcoding the sample, (8) mixing the samples and adjusting the concentration, (9) sequencing the sample. The workflow may comprise multiple sub-steps that contain one of the listed steps (*e.g.* step (2) of preparing the library step could entail three enzymatic steps (blunt ending, dA tailing and adaptor ligation) and three purification steps). Steps of the workflow may be combined, divided up or performed in different order (*e.g.* bar coding and pooling of samples).

[0354] It is important to note that the amplification of a library can be performed in such a way that it is biased to amplify short fragments more efficiently. In this manner it is possible to preferentially amplify shorter sequences, *e.g.* mono-nucleosomal DNA fragments as the cell free fetal DNA (of placental origin) found in the circulation of pregnant women. Note that PCR assays can have the tags, for example sequencing tags, (usually a truncated form of 15-25 bases). After multiplexing, PCR multiplexes of a sample are pooled and then the tags are completed (including bar coding) by a tag-specific PCR (could also be done by ligation). Also, the full sequencing tags can be added in the same reaction as the multiplexing. In the first cycles targets may be amplified with the target specific primers, subsequently the tag-specific primers take

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over to complete the SQ-adaptor sequence. The PCR primers may carry no tags. The sequencing tags may be appended to the amplification products by ligation.

[0355] In an embodiment, highly multiplex PCR followed by evaluation of amplified material by clonal sequencing may be used for various applications such as the detection of fetal aneuploidy. Whereas traditional multiplex PCRs evaluate up to fifty loci simultaneously, the approach described herein may be used to enable simultaneous evaluation of more than 50 loci simultaneously, more than 100 loci simultaneously, more than 500 loci simultaneously, more than 1,000 loci simultaneously, more than 5,000 loci simultaneously, more than 10,000 loci simultaneously, more than 50,000 loci simultaneously, and more than 100,000 loci simultaneously. Experiments have shown that up to, including and more than 10,000 distinct loci can be evaluated simultaneously, in a single reaction, with sufficiently good efficiency and specificity to make non-invasive prenatal aneuploidy diagnoses and/or copy number calls with high accuracy. Assays may be combined in a single reaction with the entirety of a sample such as a cfDNA sample isolated from maternal plasma, a fraction thereof, or a further processed derivative of the cfDNA sample. The sample (*e.g.*, cfDNA or derivative) may also be split into multiple parallel multiplex reactions. The optimum sample splitting and multiplex is determined by trading off various performance specifications. Due to the limited amount of material, splitting the sample into multiple fractions can introduce sampling noise, handling time, and increase the possibility of error. Conversely, higher multiplexing can result in greater amounts of spurious amplification and greater inequalities in amplification both of which can reduce test performance.

[0356] Two crucial related considerations in the application of the methods described herein are the limited amount of original sample (*e.g.*, plasma) and the number of original molecules in that material from which allele frequency or other measurements are obtained. If the number of original molecules falls below a certain level, random sampling noise becomes significant, and can affect the accuracy of the test. Typically, data of sufficient quality for making non-invasive prenatal aneuploidy diagnoses can be obtained if measurements are made on a sample comprising the equivalent of 500-1000 original molecules per target locus. There are a number of ways of increasing the number of distinct measurements, for example increasing the sample volume. Each manipulation applied to the sample also potentially results in losses of material. It is essential to characterize losses incurred by various manipulations and avoid, or as necessary

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improve yield of certain manipulations to avoid losses that could degrade performance of the test.

[0357] In an embodiment, it is possible to mitigate potential losses in subsequent steps by amplifying all or a fraction of the original sample (*e.g.*, cfDNA sample). Various methods are available to amplify all of the genetic material in a sample, increasing the amount available for downstream procedures. In an embodiment, ligation mediated PCR (LM-PCR) DNA fragments are amplified by PCR after ligation of either one distinct adaptors, two distinct adapters, or many distinct adaptors. In an embodiment, multiple displacement amplification (MDA) phi-29 polymerase is used to amplify all DNA isothermally. In DOP-PCR and variations, random priming is used to amplify the original material DNA. Each method has certain characteristics such as uniformity of amplification across all represented regions of the genome, efficiency of capture and amplification of original DNA, and amplification performance as a function of the length of the fragment.

[0358] In an embodiment LM-PCR may be used with a single heteroduplexed adaptor having a 3-prime tyrosine. The heteroduplexed adaptor enables the use of a single adaptor molecule that may be converted to two distinct sequences on 5-prime and 3-prime ends of the original DNA fragment during the first round of PCR. In an embodiment, it is possible to fractionate the amplified library by size separations, or products such as AMPURE, TASS or other similar methods. Prior to ligation, sample DNA may be blunt ended, and then a single adenosine base is added to the 3-prime end. Prior to ligation the DNA may be cleaved using a restriction enzyme or some other cleavage method. During ligation the 3-prime adenosine of the sample fragments and the complementary 3-prime tyrosine overhang of adaptor can enhance ligation efficiency. The extension step of the PCR amplification may be limited from a time standpoint to reduce amplification from fragments longer than about 200 bp, about 300 bp, about 400 bp, about 500 bp or about 1,000 bp. Since longer DNA found in the maternal plasma is nearly exclusively maternal, this may result in the enrichment of fetal DNA by 10-50% and improvement of test performance. A number of reactions were run using conditions as specified by commercially available kits; the resulted in successful ligation of fewer than 10% of sample DNA molecules. A series of optimizations of the reaction conditions for this improved ligation to approximately 70%.

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Mini-PCR

[0359] The following Mini-PCR method is desirable for samples containing short nucleic acids, digested nucleic acids, or fragmented nucleic acids, such as cfDNA. Traditional PCR assay design results in significant losses of distinct fetal molecules, but losses can be greatly reduced by designing very short PCR assays, termed mini-PCR assays. Fetal cfDNA in maternal serum is highly fragmented and the fragment sizes are distributed in approximately a Gaussian fashion with a mean of 160 bp, a standard deviation of 15 bp, a minimum size of about 100 bp, and a maximum size of about 220 bp. The distribution of fragment start and end positions with respect to the targeted polymorphisms, while not necessarily random, vary widely among individual targets and among all targets collectively and the polymorphic site of one particular target locus may occupy any position from the start to the end among the various fragments originating from that locus. Note that the term mini-PCR may equally well refer to normal PCR with no additional restrictions or limitations.

[0360] During PCR, amplification will only occur from template DNA fragments comprising both forward and reverse primer sites. Because fetal cfDNA fragments are short, the likelihood of both primer sites being present the likelihood of a fetal fragment of length L comprising both the forward and reverse primers sites is ratio of the length of the amplicon to the length of the fragment. Under ideal conditions, assays in which the amplicon is 45, 50, 55, 60, 65, or 70 bp will successfully amplify from 72%, 69%, 66%, 63%, 59%, or 56%, respectively, of available template fragment molecules. The amplicon length is the distance between the 5-prime ends of the forward and reverse priming sites. Amplicon length that is shorter than typically used by those known in the art may result in more efficient measurements of the desired polymorphic loci by only requiring short sequence reads. In an embodiment, a substantial fraction of the amplicons should be less than 100 bp, less than 90 bp, less than 80 bp, less than 70 bp, less than 65 bp, less than 60 bp, less than 55 bp, less than 50 bp, or less than 45 bp.

[0361] Note that in methods known in the prior art, short assays such as those described herein are usually avoided because they are not required and they impose considerable constraint on primer design by limiting primer length, annealing characteristics, and the distance between the forward and reverse primer.

[0362] Also note that there is the potential for biased amplification if the 3-prime end of the either primer is within roughly 1-6 bases of the polymorphic site. This single base difference at the site of initial polymerase binding can result in preferential amplification of one allele, which

can alter observed allele frequencies and degrade performance. All of these constraints make it very challenging to identify primers that will amplify a particular locus successfully and furthermore, to design large sets of primers that are compatible in the same multiplex reaction. In an embodiment, the 3' end of the inner forward and reverse primers are designed to hybridize to a region of DNA upstream from the polymorphic site, and separated from the polymorphic site by a small number of bases. Ideally, the number of bases may be between 6 and 10 bases, but may equally well be between 4 and 15 bases, between three and 20 bases, between two and 30 bases, or between 1 and 60 bases, and achieve substantially the same end.

[0363] Multiplex PCR may involve a single round of PCR in which all targets are amplified or it may involve one round of PCR followed by one or more rounds of nested PCR or some variant of nested PCR. Nested PCR consists of a subsequent round or rounds of PCR amplification using one or more new primers that bind internally, by at least one base pair, to the primers used in a previous round. Nested PCR reduces the number of spurious amplification targets by amplifying, in subsequent reactions, only those amplification products from the previous one that have the correct internal sequence. Reducing spurious amplification targets improves the number of useful measurements that can be obtained, especially in sequencing. Nested PCR typically entails designing primers completely internal to the previous primer binding sites, necessarily increasing the minimum DNA segment size required for amplification. For samples such as maternal plasma cfDNA, in which the DNA is highly fragmented, the larger assay size reduces the number of distinct cfDNA molecules from which a measurement can be obtained. In an embodiment, to offset this effect, one may use a partial nesting approach where one or both of the second round primers overlap the first binding sites extending internally some number of bases to achieve additional specificity while minimally increasing in the total assay size.

[0364] In an embodiment, a multiplex pool of PCR assays are designed to amplify potentially heterozygous SNP or other polymorphic or non-polymorphic loci on one or more chromosomes and these assays are used in a single reaction to amplify DNA. The number of PCR assays may be between 50 and 200 PCR assays, between 200 and 1,000 PCR assays, between 1,000 and 5,000 PCR assays, or between 5,000 and 20,000 PCR assays (50 to 200-plex, 200 to 1,000-plex, 1,000 to 5,000-plex, 5,000 to 20,000-plex, more than 20,000-plex respectively). In an embodiment, a multiplex pool of about 10,000 PCR assays (10,000-plex) are designed to amplify potentially heterozygous SNP loci on chromosomes X, Y, 13, 18, and 21 and 1 or 2 and these assays are used in a single reaction to amplify cfDNA obtained from a material plasma sample,

chorion villus samples, amniocentesis samples, single or a small number of cells, other bodily fluids or tissues, cancers, or other genetic matter. The SNP frequencies of each locus may be determined by clonal or some other method of sequencing of the amplicons. Statistical analysis of the allele frequency distributions or ratios of all assays may be used to determine if the sample contains a trisomy of one or more of the chromosomes included in the test. In another embodiment the original cfDNA samples is split into two samples and parallel 5,000-plex assays are performed. In another embodiment the original cfDNA samples is split into n samples and parallel (~10,000/n)-plex assays are performed where n is between 2 and 12, or between 12 and 24, or between 24 and 48, or between 48 and 96. Data is collected and analyzed in a similar manner to that already described. Note that this method is equally well applicable to detecting translocations, deletions, duplications, and other chromosomal abnormalities.

[0365] In an embodiment, tails with no homology to the target genome may also be added to the 3-prime or 5-prime end of any of the primers. These tails facilitate subsequent manipulations, procedures, or measurements. In an embodiment, the tail sequence can be the same for the forward and reverse target specific primers. In an embodiment, different tails may be used for the forward and reverse target specific primers. In an embodiment, a plurality of different tails may be used for different loci or sets of loci. Certain tails may be shared among all loci or among subsets of loci. For example, using forward and reverse tails corresponding to forward and reverse sequences required by any of the current sequencing platforms can enable direct sequencing following amplification. In an embodiment, the tails can be used as common priming sites among all amplified targets that can be used to add other useful sequences. In some embodiments, the inner primers may contain a region that is designed to hybridize either upstream or downstream of the targeted locus (*e.g*, a polymorphic locus). In some embodiments, the primers may contain a molecular barcode. In some embodiments, the primer may contain a universal priming sequence designed to allow PCR amplification.

[0366] In an embodiment, a 10,000-plex PCR assay pool is created such that forward and reverse primers have tails corresponding to the required forward and reverse sequences required by a high throughput sequencing instrument such as the HISEQ, GAIIX, or MYSEQ available from ILLUMINA. In addition, included 5-prime to the sequencing tails is an additional sequence that can be used as a priming site in a subsequent PCR to add nucleotide barcode sequences to the amplicons, enabling multiplex sequencing of multiple samples in a single lane of the high throughput sequencing instrument.

[0367] In an embodiment, a 10,000-plex PCR assay pool is created such that reverse primers have tails corresponding to the required reverse sequences required by a high throughput sequencing instrument. After amplification with the first 10,000-plex assay, a subsequent PCR amplification may be performed using a another 10,000-plex pool having partly nested forward primers (*e.g.* 6-bases nested) for all targets and a reverse primer corresponding to the reverse sequencing tail included in the first round. This subsequent round of partly nested amplification with just one target specific primer and a universal primer limits the required size of the assay, reducing sampling noise, but greatly reduces the number of spurious amplicons. The sequencing tags can be added to appended ligation adaptors and/or as part of PCR probes, such that the tag is part of the final amplicon.

[0368] Fetal fraction affects performance of the test. There are a number of ways to enrich the fetal fraction of the DNA found in maternal plasma. Fetal fraction can be increased by the previously described LM-PCR method already discussed as well as by a targeted removal of long maternal fragments. In an embodiment, prior to multiplex PCR amplification of the target loci, an additional multiplex PCR reaction may be carried out to selectively remove long and largely maternal fragments corresponding to the loci targeted in the subsequent multiplex PCR. Additional primers are designed to anneal a site a greater distance from the polymorphism than is expected to be present among cell free fetal DNA fragments. These primers may be used in a one cycle multiplex PCR reaction prior to multiplex PCR of the target polymorphic loci. These distal primers are tagged with a molecule or moiety that can allow selective recognition of the tagged pieces of DNA. In an embodiment, these molecules of DNA may be covalently modified with a biotin molecule that allows removal of newly formed double stranded DNA comprising these primers after one cycle of PCR. Double stranded DNA formed during that first round is likely maternal in origin. Removal of the hybrid material may be accomplish by the used of magnetic streptavidin beads. There are other methods of tagging that may work equally well. In an embodiment, size selection methods may be used to enrich the sample for shorter strands of DNA; for example those less than about 800 bp, less than about 500 bp, or less than about 300 bp. Amplification of short fragments can then proceed as usual.

[0369] The mini-PCR method described in this disclosure enables highly multiplexed amplification and analysis of hundreds to thousands or even millions of loci in a single reaction, from a single sample. At the same, the detection of the amplified DNA can be multiplexed; tens to hundreds of samples can be multiplexed in one sequencing lane by using barcoding PCR. This

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multiplexed detection has been successfully tested up to 49-plex, and a much higher degree of multiplexing is possible. In effect, this allows hundreds of samples to be genotyped at thousands of SNPs in a single sequencing run. For these samples, the method allows determination of genotype and heterozygosity rate and simultaneously determination of copy number, both of which may be used for the purpose of aneuploidy detection. This method is particularly useful in detecting aneuploidy of a gestating fetus from the free floating DNA found in maternal plasma. This method may be used as part of a method for sexing a fetus, and/or predicting the paternity of the fetus. It may be used as part of a method for mutation dosage. This method may be used for any amount of DNA or RNA, and the targeted regions may be SNPs, other polymorphic regions, non-polymorphic regions, and combinations thereof.

[0370] In some embodiments, ligation mediated universal-PCR amplification of fragmented DNA may be used. The ligation mediated universal-PCR amplification can be used to amplify plasma DNA, which can then be divided into multiple parallel reactions. It may also be used to preferentially amplify short fragments, thereby enriching fetal fraction. In some embodiments the addition of tags to the fragments by ligation can enable detection of shorter fragments, use of shorter target sequence specific portions of the primers and/or annealing at higher temperatures which reduces unspecific reactions.

[0371] The methods described herein may be used for a number of purposes where there is a target set of DNA that is mixed with an amount of contaminating DNA. In some embodiments, the target DNA and the contaminating DNA may be from individuals who are genetically related. For example, genetic abnormalities in a fetus (target) may be detected from maternal plasma which contains fetal (target) DNA and also maternal (contaminating) DNA; the abnormalities include whole chromosome abnormalities (*e.g.* aneuploidy) partial chromosome abnormalities (*e.g.* deletions, duplications, inversions, and translocations), polynucleotide polymorphisms (*e.g.* STRs), single nucleotide polymorphisms, and/or other genetic abnormalities or differences. In some embodiments, the target and contaminating DNA may be from the same individual, but where the target and contaminating DNA are different by one or more mutations, for example in the case of cancer. (see *e.g.* H. Mamon et al. *Preferential Amplification of Apoptotic DNA from Plasma: Potential for Enhancing Detection of Minor DNA Alterations in Circulating DNA*. Clinical Chemistry 54:9 (2008). In some embodiments, the DNA may be found in cell culture (apoptotic) supernatant. In some embodiments, it is possible to induce apoptosis in biological samples (*e.g.,* blood) for subsequent library preparation,

amplification and/or sequencing. A number of enabling workflows and protocols to achieve this end are presented elsewhere in this disclosure.

[0372] In some embodiments, the target DNA may originate from single cells, from samples of DNA consisting of less than one copy of the target genome, from low amounts of DNA, from DNA from mixed origin (*e.g.* pregnancy plasma: placental and maternal DNA; cancer patient plasma and tumors: mix between healthy and cancer DNA, transplantation *etc*), from other body fluids, from cell cultures, from culture supernatants, from forensic samples of DNA, from ancient samples of DNA (*e.g.* insects trapped in amber), from other samples of DNA, and combinations thereof.

[0373] In some embodiments, a short amplicon size may be used. Short amplicon sizes are especially suited for fragmented DNA (see *e.g.* A. Sikora, et sl. Detection of increased amounts of cell-free fetal DNA with short PCR amplicons. Clin Chem. 2010 Jan;56(1):136-8.)

[0374] The use of short amplicon sizes may result in some significant benefits. Short amplicon sizes may result in optimized amplification efficiency. Short amplicon sizes typically produce shorter products, therefore there is less chance for nonspecific priming. Shorter products can be clustered more densely on sequencing flow cell, as the clusters will be smaller. Note that the methods described herein may work equally well for longer PCR amplicons. Amplicon length may be increased if necessary, for example, when sequencing larger sequence stretches. Experiments with 146-plex targeted amplification with assays of 100 bp to 200 bp length as first step in a nested-PCR protocol were run on single cells and on genomic DNA with positive results.

[0375] In some embodiments, the methods described herein may be used to amplify and/or detect SNPs, copy number, nucleotide methylation, mRNA levels, other types of RNA expression levels, other genetic and/or epigenetic features. The mini-PCR methods described herein may be used along with next-generation sequencing; it may be used with other downstream methods such as microarrays, counting by digital PCR, real-time PCR, Mass-spectrometry analysis etc.

[0376] In some embodiment, the mini-PCR amplification methods described herein may be used as part of a method for accurate quantification of minority populations. It may be used for absolute quantification using spike calibrators. It may be used for mutation / minor allele quantification through very deep sequencing, and may be run in a highly multiplexed fashion. It may be used for standard paternity and identity testing of relatives or ancestors, in human,

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animals, plants or other creatures. It may be used for forensic testing. It may be used for rapid genotyping and copy number analysis (CN), on any kind of material, *e.g.* amniotic fluid and CVS, sperm, product of conception (POC). It may be used for single cell analysis, such as genotyping on samples biopsied from embryos. It may be used for rapid embryo analysis (within less than one, one, or two days of biopsy) by targeted sequencing using min-PCR.

[0377] In some embodiments, it may be used for tumor analysis: tumor biopsies are often a mixture of health and tumor cells. Targeted PCR allows deep sequencing of SNPs and loci with close to no background sequences. It may be used for copy number and loss of heterozygosity analysis on tumor DNA. Said tumor DNA may be present in many different body fluids or tissues of tumor patients. It may be used for detection of tumor recurrence, and/or tumor screening. It may be used for quality control testing of seeds. It may be used for breeding, or fishing purposes. Note that any of these methods could equally well be used targeting non-polymorphic loci for the purpose of ploidy calling.

[0378] Some literature describing some of the fundamental methods that underlie the methods disclosed herein include: (1) Wang HY, Luo M, Tereshchenko IV, Frikker DM, Cui X, Li JY, Hu G, Chu Y, Azaro MA, Lin Y, Shen L, Yang Q, Kambouris ME, Gao R, Shih W, Li H. Genome Res. 2005 Feb;15(2):276-83. Department of Molecular Genetics, Microbiology and Immunology/The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA. (2) High-throughput genotyping of single nucleotide polymorphisms with high sensitivity. Li H, Wang HY, Cui X, Luo M, Hu G, Greenawalt DM, Tereshchenko IV, Li JY, Chu Y, Gao R. Methods Mol Biol. 2007;396 - PubMed PMID: 18025699. (3) A method comprising multiplexing of an average of 9 assays for sequencing is described in: Nested Patch PCR enables highly multiplexed mutation discovery in candidate genes. Varley KE, Mitra RD. Genome Res. 2008 Nov;18(11):1844-50. Epub 2008 Oct 10. Note that the methods disclosed herein allow multiplexing of orders of magnitude more than in the above references.

Targeted PCR Variants - Nesting

[0379] There are many workflows that are possible when conducting PCR; some workflows typical to the methods disclosed herein are described. The steps outlined herein are not meant to exclude other possible steps nor does it imply that any of the steps described herein are required

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for the method to work properly. A large number of parameter variations or other modifications are known in the literature, and may be made without affecting the essence of the invention. One particular generalized workflow is given below followed by a number of possible variants. The variants typically refer to possible secondary PCR reactions, for example different types of nesting that may be done (step 3). It is important to note that variants may be done at different times, or in different orders than explicitly described herein. Examples that use polymorphic loci for illustration can be readily adapted for the amplification of nonpolymorphic loci if desired.

[0380] The DNA in the sample may have ligation adapters, often referred to as library tags or ligation adaptor tags (LTs), appended, where the ligation adapters contain a universal priming sequence, followed by a universal amplification. In an embodiment, this may be done using a standard protocol designed to create sequencing libraries after fragmentation. In an embodiment, the DNA sample can be blunt ended, and then an A can be added at the 3' end. A Y-adaptor with a T-overhang can be added and ligated. In some embodiments, other sticky ends can be used other than an A or T overhang. In some embodiments, other adaptors can be added, for example looped ligation adaptors. In some embodiments, the adaptors may have tag designed for PCR amplification.

[0381] Specific Target Amplification (STA): Pre-amplification of hundreds to thousands to tens of thousands and even hundreds of thousands of targets may be multiplexed in one reaction volume. STA is typically run from 10 to 30 cycles, though it may be run from 5 to 40 cycles, from 2 to 50 cycles, and even from 1 to 100 cycles. Primers may be tailed, for example for a simpler workflow or to avoid sequencing of a large proportion of dimers. Note that typically, dimers of both primers carrying the same tag will not be amplified or sequenced efficiently. In some embodiments, between 1 and 10 cycles of PCR may be carried out; in some embodiments between 10 and 20 cycles of PCR may be carried out; in some embodiments between 20 and 30 cycles of PCR may be carried out; in some embodiments between 30 and 40 cycles of PCR may be carried out; in some embodiments more than 40 cycles of PCR may be carried out. The amplification may be a linear amplification. The number of PCR cycles may be optimized to result in an optimal depth of read (DOR) profile. Different DOR profiles may be desirable for different purposes. In some embodiments, a more even distribution of reads between all assays is desirable; if the DOR is too small for some assays, the stochastic noise can be too high for the

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data to be too useful, while if the depth of read is too high, the marginal usefulness of each additional read is relatively small.

[0382] Primer tails may improve the detection of fragmented DNA from universally tagged libraries. If the library tag and the primer-tails contain a homologous sequence, hybridization can be improved (for example, melting temperature (T_M) is lowered) and primers can be extended if only a portion of the primer target sequence is in the sample DNA fragment. In some embodiments, 13 or more target specific base pairs may be used. In some embodiments, 10 to 12 target specific base pairs may be used. In some embodiments, 8 to 9 target specific base pairs may be used. In some embodiments, 6 to 7 target specific base pairs may be used. In some embodiments, STA may be performed on pre-amplified DNA, *e.g.* MDA, RCA, other whole genome amplifications, or adaptor-mediated universal PCR. In some embodiments, STA may be performed on samples that are enriched or depleted of certain sequences and populations, *e.g.* by size selection, target capture, directed degradation.

[0383] In some embodiments, it is possible to perform secondary multiplex PCRs or primer extension reactions to increase specificity and reduce undesirable products. For example, full nesting, semi-nesting, hemi-nesting, and/or subdividing into parallel reactions of smaller assay pools are all techniques that may be used to increase specificity. Experiments have shown that splitting a sample into three 400-plex reactions resulted in product DNA with greater specificity than one 1,200-plex reaction with exactly the same primers. Similarly, experiments have shown that splitting a sample into four 2,400-plex reactions resulted in product DNA with greater specificity than one 9,600-plex reaction with exactly the same primers. In an embodiment, it is possible to use target-specific and tag specific primers of the same and opposing directionality.

[0384] In some embodiments, it is possible to amplify a DNA sample (dilution, purified or otherwise) produced by an STA reaction using tag-specific primers and "universal amplification", *i.e.* to amplify many or all pre-amplified and tagged targets. Primers may contain additional functional sequences, *e.g.* barcodes, or a full adaptor sequence necessary for sequencing on a high throughput sequencing platform.

[0385] These methods may be used for analysis of any sample of DNA, and are especially useful when the sample of DNA is particularly small, or when it is a sample of DNA where the DNA originates from more than one individual, such as in the case of maternal plasma. These methods may be used on DNA samples such as a single or small number of cells, genomic DNA, plasma DNA, amplified plasma libraries, amplified apoptotic supernatant libraries, or other samples of

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mixed DNA. In an embodiment, these methods may be used in the case where cells of different genetic constitution may be present in a single individual, such as with cancer or transplants. In an embodiment, some of the DNA is from the recipient of a transplant (such as recipient cell-free or cellular DNA) and some of the DNA is from the donor of the transplant (such as cell-free or cellular DNA from the transplant). In an embodiment, the method is used to amplify one or more loci that differ between the recipient and the donor (such as loci for which a different combination of alleles are present in the recipient compared to the donor). In some embodiments, the recipient is homozygous for a first allele (such as AA) and the donor is homozygous for a second allele (such as BB) or is heterozygous with the first allele and a second allele (such as AB) at one or more loci. In some embodiments, the method is used to measure the absolute or relative amount of DNA from the donor of the transplant (such as cell-free or cellular DNA from the transplant). In some embodiments, this method is used to prognose, diagnose, detect, or monitor a transplant status or outcome, such as transplant rejection, tolerance, non-rejection based allograft injury, transplant function, transplant survival, chronic transplant injury, or tittering of pharmacological immunosuppression.

Protocol variants (variants and/or additions to the workflow above)

[0386] *Direct multiplexed mini-PCR*: Specific target amplification (STA) of a plurality of target sequences with tagged primers is shown in FIG. 1. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with PCR primers hybridized. 104 denotes the final PCR product. In some embodiments, STA may be done on more than 100, more than 200, more than 500, more than 1,000, more than 2,000, more than 5,000, more than 10,000, more than 20,000, more than 50,000, more than 100,000 or more than 200,000 targets. In a subsequent reaction, tag-specific primers amplify all target sequences and lengthen the tags to include all necessary sequences for sequencing, including sample indexes. In an embodiment, primers may not be tagged or only certain primers may be tagged. Sequencing adaptors may be added by conventional adaptor ligation. In an embodiment, the initial primers may carry the tags.

[0387] In an embodiment, primers are designed so that the length of DNA amplified is unexpectedly short. Prior art demonstrates that ordinary people skilled in the art typically design

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100+ bp amplicons. In an embodiment, the amplicons may be designed to be less than 80 bp. In an embodiment, the amplicons may be designed to be less than 70 bp. In an embodiment, the amplicons may be designed to be less than 60 bp. In an embodiment, the amplicons may be designed to be less than 50 bp. In an embodiment, the amplicons may be designed to be less than 45 bp. In an embodiment, the amplicons may be designed to be less than 40 bp. In an embodiment, the amplicons may be designed to be less than 35 bp. In an embodiment, the amplicons may be designed to be between 40 and 65 bp.

[0388] An experiment was performed using this protocol using 1200-plex amplification. Both genomic DNA and pregnancy plasma were used; about 70% of sequence reads mapped to targeted sequences. Details are given elsewhere in this document. Sequencing of a 1042-plex without design and selection of assays resulted in >99% of sequences being primer dimer products.

[0389] *Sequential PCR*: After STA1 multiple aliquots of the product may be amplified in parallel with pools of reduced complexity with the same primers. The first amplification can give enough material to split. This method is especially good for small samples, for example those that are about 6-100 pg, about 100 pg to 1 ng, about 1 ng to 10 ng, or about 10 ng to 100 ng. The protocol was performed with 1200-plex into three 400-plexes. Mapping of sequencing reads increased from around 60 to 70 % in the 1200-plex alone to over 95%.

[0390] *Semi-nested mini-PCR*: (see FIG. 2) After STA 1 a second STA is performed comprising a multiplex set of internal nested Forward primers (103 B, 105 b) and one (or few) tag-specific Reverse primers (103 A). 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with Forward primer B and Reverse Primer A hybridized. 104 denotes the PCR product from 103. 105 denotes the product from 104 with nested Forward primer b hybridized, and Reverse tag A already part of the molecule from the PCR that occurred between 103 and 104. 106 denotes the final PCR product. With this workflow usually greater than 95% of sequences map to the intended targets. The nested primer may overlap with the outer Forward primer sequence but introduces additional 3'-end bases. In some embodiments it is possible to use between one and 20 extra 3' bases. Experiments have shown that using 9 or more extra 3' bases in a 1200-plex designs works well. As readily apparent, the primers for the second STA

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can alternatively be considered a multiplex set of internal nested Reverse primers and one (or a few) tag-specific Forward primers.

[0391] *Fully nested mini-PCR*: (see FIG. 3) After STA step 1, it is possible to perform a second multiplex PCR (or parallel m.p. PCRs of reduced complexity) with two nested primers carrying tags (A, a, B, b). 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with Forward primer B and Reverse Primer A hybridized. 104 denotes the PCR product from 103. 105 denotes the product from 104 with nested Forward primer b and nested Reverse primer a hybridized. 106 denotes the final PCR product. In some embodiments, it is possible to use two full sets of primers. Experiments using a fully nested mini-PCR protocol were used to perform 146-plex amplification on single and three cells without step 102 of appending universal ligation adaptors and amplifying.

[0392] *Hemi-nested mini-PCR*: (see FIG. 4) It is possible to use target DNA that has and adaptors at the fragment ends. STA is performed comprising a multiplex set of Forward primers (B) and one (or few) tag-specific Reverse primers (A). A second STA can be performed using a universal tag-specific Forward primer and target specific Reverse primer. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with Reverse Primer A hybridized. 104 denotes the PCR product from 103 that was amplified using Reverse primer A and ligation adaptor tag primer LT. 105 denotes the product from 104 with Forward primer B hybridized. 106 denotes the final PCR product. In this workflow, target specific Forward and Reverse primers are used in separate reactions, thereby reducing the complexity of the reaction and preventing dimer formation of forward and reverse primers. Note that in this example, primers A and B may be considered to be first primers, and primers 'a' and 'b' may be considered to be inner primers. This method is a big improvement on direct PCR as it is as good as direct PCR, but it avoids primer dimers. After first round of hemi nested protocol one typically sees ~99% non-targeted DNA, however, after second round there is typically a big improvement. As readily apparent, the primers for the first STA can be considered a multiplex set of Reverse primers and one (or few) tag-specific Forward primers, and the primers for the second STA can be considered a universal tag-specific Reverse primer and target specific Forward primer(s).

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[0393] *Triply hemi-nested mini-PCR*: (see FIG. 5) It is possible to use target DNA that has and adaptor at the fragment ends. STA is performed comprising a multiplex set of Forward primers (B) and one (or few) tag-specific Reverse primers (A) and (a). A second STA can be performed using a universal tag-specific Forward primer and target specific Reverse primer. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with Reverse Primer A hybridized. 104 denotes the PCR product from 103 that was amplified using Reverse primer A and ligation adaptor tag primer LT. 105 denotes the product from 104 with Forward primer B hybridized. 106 denotes the PCR product from 105 that was amplified using Reverse primer A and Forward primer B. 107 denotes the product from 106 with Reverse primer 'a' hybridized. 108 denotes the final PCR product. Note that in this example, primers 'a' and B may be considered to be inner primers, and A may be considered to be a first primer. Optionally, both A and B may be considered to be first primers, and 'a' may be considered to be an inner primer. The designation of reverse and forward primers may be switched. In this workflow, target specific Forward and Reverse primers are used in separate reactions, thereby reducing the complexity of the reaction and preventing dimer formation of forward and reverse primers. This method is a big improvement on direct PCR as it is as good as direct PCR, but it avoids primer dimers. After first round of hemi nested protocol one typically sees ~99% non-targeted DNA, however, after second round there is typically a big improvement.

[0394] *One-sided nested mini-PCR*: (see FIG. 6) It is possible to use target DNA that has an adaptor at the fragment ends. STA may also be performed with a multiplex set of nested Forward primers and using the ligation adapter tag as the Reverse primer. A second STA may then be performed using a set of nested Forward primers and a universal Reverse primer. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA that has been universally amplified with Forward Primer A hybridized. 104 denotes the PCR product from 103 that was amplified using Forward primer A and ligation adaptor tag Reverse primer LT. 105 denotes the product from 104 with nested Forward primer a hybridized. 106 denotes the final PCR product. This method can detect shorter target sequences than standard PCR by using overlapping primers in the first and second STAs. The method is typically performed off a sample of DNA that has already undergone STA step 1 above –

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appending of universal tags and amplification; the two nested primers are only on one side, other side uses the library tag. The method was performed on libraries of apoptotic supernatants and pregnancy plasma. With this workflow around 60% of sequences mapped to the intended targets. Note that reads that contained the reverse adaptor sequence were not mapped, so this number is expected to be higher if those reads that contain the reverse adaptor sequence are mapped

[0395] *One-sided mini-PCR*: It is possible to use target DNA that has an adaptor at the fragment ends (see FIG. 7). STA may be performed with a multiplex set of Forward primers and one (or few) tag-specific Reverse primer. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA with Forward Primer A hybridized. 104 denotes the PCR product from 103 that was amplified using Forward primer A and ligation adaptor tag Reverse primer LT, and which is the final PCR product. This method can detect shorter target sequences than standard PCR. However it may be relatively unspecific, as only one target specific primer is used. This protocol is effectively half of the one sided nested mini PCR

[0396] *Reverse semi-nested mini-PCR*: It is possible to use target DNA that has an adaptor at the fragment ends (see FIG. 8). STA may be performed with a multiplex set of Forward primers and one (or few) tag-specific Reverse primer. 101 denotes double stranded DNA with a polymorphic locus of interest at X. 102 denotes the double stranded DNA with ligation adaptors added for universal amplification. 103 denotes the single stranded DNA with Reverse Primer B hybridized. 104 denotes the PCR product from 103 that was amplified using Reverse primer B and ligation adaptor tag Forward primer LT. 105 denotes the PCR product 104 with hybridized Forward Primer A, and inner Reverse primer 'b'. 106 denotes the PCR product that has been amplified from 105 using Forward primer A and Reverse primer 'b', and which is the final PCR product. This method can detect shorter target sequences than standard PCR.

[0397] There also may be more variants that are simply iterations or combinations of the above methods such as doubly nested PCR, where three sets of primers are used. Another variant is one-and-a-half sided nested mini-PCR, where STA may also be performed with a multiplex set of nested Forward primers and one (or few) tag-specific Reverse primer.

[0398] Note that in all of these variants, the identity of the Forward primer and the Reverse primer may be interchanged. Note that in some embodiments, the nested variant can equally well be run without the initial library preparation that comprises appending the adapter tags, and a

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universal amplification step. Note that in some embodiments, additional rounds of PCR may be included, with additional Forward and/or Reverse primers and amplification steps; these additional steps may be particularly useful if it is desirable to further increase the percent of DNA molecules that correspond to the targeted loci.

Nesting Workflows

[0399] There are many ways to perform the amplification, with different degrees of nesting, and with different degrees of multiplexing. In FIG. 9, a flow chart is given with some of the possible workflows. Note that the use of 10,000-plex PCR is only meant to be an example; these flow charts would work equally well for other degrees of multiplexing.

Looped ligation adaptors

[0400] When adding universal tagged adaptors for example for the purpose of making a library for sequencing, there are a number of ways to ligate adaptors. One way is to blunt end the sample DNA, perform A-tailing, and ligate with adaptors that have a T-overhang. There are a number of other ways to ligate adaptors. There are also a number of adaptors that can be ligated. For example, a Y-adaptor can be used where the adaptor consists of two strands of DNA where one strand has a double strand region, and a region specified by a forward primer region, and where the other strand specified by a double strand region that is complementary to the double strand region on the first strand, and a region with a reverse primer. The double stranded region, when annealed, may contain a T-overhang for the purpose of ligating to double stranded DNA with an A overhang.

[0401] In an embodiment, the adaptor can be a loop of DNA where the terminal regions are complementary, and where the loop region contains a forward primer tagged region (LFT), a reverse primer tagged region (LRT), and a cleavage site between the two (See FIG. 10). 101 refers to the double stranded, blunt ended target DNA. 102 refers to the A-tailed target DNA. 103 refers to the looped ligation adaptor with T overhang 'T' and the cleavage site 'Z'. 104 refers to the target DNA with appended looped ligation adaptors. 105 refers to the target DNA with the ligation adaptors appended cleaved at the cleavage site. LFT refers to the ligation adaptor Forward tag, and the LRT refers to the ligation adaptor Reverse tag. The complementary region may end on a T overhang, or other feature that may be used for ligation to the target DNA.

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The cleavage site may be a series of uracils for cleavage by UNG, or a sequence that may be recognized and cleaved by a restriction enzyme or other method of cleavage or just a basic amplification. These adaptors can be uses for any library preparation, for example, for sequencing. These adaptors can be used in combination with any of the other methods described herein, for example the mini-PCR amplification methods.

Internally Tagged Primers

[0402] When using sequencing to determine the allele present at a given polymorphic locus, the sequence read typically begins upstream of the primer binding site (a), and then to the polymorphic site (X). Tags are typically configured as shown in FIG. 11, left. 101 refers to the single stranded target DNA with polymorphic locus of interest 'X', and primer 'a' with appended tag 'b'. In order to avoid nonspecific hybridization, the primer binding site (region of target DNA complementary to 'a') is typically 18 to 30 bp in length. Sequence tag 'b' is typically about 20 bp; in theory these can be any length longer than about 15 bp, though many people use the primer sequences that are sold by the sequencing platform company. The distance 'd' between 'a' and 'X' may be at least 2 bp so as to avoid allele bias. When performing multiplexed PCR amplification using the methods disclosed herein or other methods, where careful primer design is necessary to avoid excessive primer primer interaction, the window of allowable distance 'd' between 'a' and 'X' may vary quite a bit: from 2 bp to 10 bp, from 2 bp to 20 bp, from 2 bp to 30 bp, or even from 2 bp to more than 30 bp. Therefore, when using the primer configuration shown in FIG. 11, left, sequence reads must be a minimum of 40 bp to obtain reads long enough to measure the polymorphic locus, and depending on the lengths of 'a' and 'd' the sequence reads may need to be up to 60 or 75 bp. Usually, the longer the sequence reads, the higher the cost and time of sequencing a given number of reads, therefore, minimizing the necessary read length can save both time and money. In addition, since, on average, bases read earlier on the read are read more accurately than those read later on the read, decreasing the necessary sequence read length can also increase the accuracy of the measurements of the polymorphic region.

[0403] In an embodiment, termed internally tagged primers, the primer binding site (a) is split in to a plurality of segments (a', a", a'"….), and the sequence tag (b) is on a segment of DNA that is in the middle of two of the primer binding sites, as shown in FIG. 11, 103. This configuration allows the sequencer to make shorter sequence reads. In an embodiment, a' + a"

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should be at least about 18 bp, and can be as long as 30, 40, 50, 60, 80, 100 or more than 100 bp. In an embodiment, a" should be at least about 6 bp, and in an embodiment is between about 8 and 16 bp. All other factors being equal, using the internally tagged primers can cut the length of the sequence reads needed by at least 6 bp, as much as 8 bp, 10 bp, 12 bp, 15 bp, and even by as many as 20 or 30 bp. This can result in a significant money, time and accuracy advantage. An example of internally tagged primers is given in FIG. 12.

Primers with ligation adaptor binding region

[0404] One issue with fragmented DNA is that since it is short in length, the chance that a polymorphism is close to the end of a DNA strand is higher than for a long strand (*e.g.* 101, FIG. 10). Since PCR capture of a polymorphism requires a primer binding site of suitable length on both sides of the polymorphism, a significant number of strands of DNA with the targeted polymorphism will be missed due to insufficient overlap between the primer and the targeted binding site. In an embodiment, the target DNA 101 can have ligation adaptors appended 102, and the target primer 103 can have a region (cr) that is complementary to the ligation adaptor tag (lt) appended upstream of the designed binding region (a) (see FIG. 13); thus in cases where the binding region (region of 101 that is complementary to a) is shorter than the 18 bp typically required for hybridization, the region (cr) on the primer than is complementary to the library tag is able to increase the binding energy to a point where the PCR can proceed. Note that any specificity that is lost due to a shorter binding region can be made up for by other PCR primers with suitably long target binding regions. Note that this embodiment can be used in combination with direct PCR, or any of the other methods described herein, such as nested PCR, semi nested PCR, hemi nested PCR, one sided nested or semi or hemi nested PCR, or other PCR protocols.

[0405] When using the sequencing data to determine ploidy in combination with an analytical method that involves comparing the observed allele data to the expected allele distributions for various hypotheses, each additional read from alleles with a low depth of read will yield more information than a read from an allele with a high depth of read. Therefore, ideally, one would wish to see uniform depth of read (DOR) where each locus will have a similar number of representative sequence reads. Therefore, it is desirable to minimize the DOR variance. In an embodiment, it is possible to decrease the coefficient of variance of the DOR (this may be defined as the standard deviation of the DOR / the average DOR) by increasing the annealing times. In some embodiments the annealing temperatures may be longer than 2 minutes, longer

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than 4 minutes, longer than ten minutes, longer than 30 minutes, and longer than one hour, or even longer. Since annealing is an equilibrium process, there is no limit to the improvement of DOR variance with increasing annealing times. In an embodiment, increasing the primer concentration may decrease the DOR variance.

Exemplary Amplification Methods

[0406] Improved PCR amplification methods have also been developed that minimize or prevent interference due to the amplification of nearby or adjacent target loci in the same reaction volume (such as part of the sample multiplex PCR reaction that simultaneously amplifies all the target loci) (see, USSN 61/982,245, filed April 21, 2014; USSN 61/987,407, filed May 1, 2014, and USSN 62/066,514, filed Oct. 21, 2014, which are each hereby incorporated by reference in its entirety). These methods can be used to simultaneously amplify nearby or adjacent target loci, which is faster and cheaper than having to separate nearby target loci into different reaction volumes so that they can be amplified separately to avoid interference. In particular embodiments, these methods are used to tile a region such that the amplicons include all the nucleotides in the region (such as an exon or all the exons of a gene such as cystic fibrosis).

[0407] In some embodiments, the amplification of target loci is performed using a polymerase (*e.g.*, a DNA polymerase, RNA polymerase, or reverse transcriptase) with low 5′→ 3′ exonuclease and/or low strand displacement activity. In some embodiments, the low level of 5′→ 3′ exonuclease reduces or prevents the degradation of a nearby primer (*e.g.*, an unextended primer or a primer that has had one or more nucleotides added to during primer extension). In some embodiments, the low level of strand displacement activity reduces or prevents the displacement of a nearby primer (*e.g.*, an unextended primer or a primer that has had one or more nucleotides added to it during primer extension). In some embodiments, target loci that are adjacent to each other (*e.g.*, no bases between the target loci) or nearby (*e.g.*, loci are within 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 base) are amplified. In some embodiments, the 3′ end of one locus is within 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 base of the 5′ end of next downstream locus.

[0408] In some embodiments, at least 100, 200, 500, 750, 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci are amplified, such as by the simultaneous amplification in one reaction

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volume In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the amplified products are target amplicons. In various embodiments, the amount of amplified products that are target amplicons is between 50 to 99.5%, such as between 60 to 99%, 70 to 98%, 80 to 98%, 90 to 99.5%, or 95 to 99.5%, inclusive. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the target loci are amplified (*e.g*, amplified at least 5, 10, 20, 30, 50, or 100-fold compared to the amount prior to amplification), such as by the simultaneous amplification in one reaction volume. In various embodiments, the amount target loci that are amplified (*e.g*, amplified at least 5, 10, 20, 30, 50, or 100-fold compared to the amount prior to amplification) is between 50 to 99.5%, such as between 60 to 99%, 70 to 98%, 80 to 99%, 90 to 99.5%, 95 to 99.9%, or 98 to 99.99% inclusive. In some embodiments, fewer non-target amplicons are produced, such as fewer amplicons formed from a forward primer from a first primer pair and a reverse primer from a second primer pair. Such undesired non-target amplicons can be produced using prior amplification methods if, *e.g.*, the reverse primer from the first primer pair and/or the forward primer from the second primer pair are degraded and/or displaced.

[0409] In some embodiments, these methods allows longer extension times to be used since the polymerase bound to a primer being extended is less likely to degrade and/or displace a nearby primer (such as the next downstream primer) given the low 5´→ 3´ exonuclease and/or low strand displacement activity of the polymerase. In various embodiments, reaction conditions (such as the extension time and temperature) are used such that the extension rate of the polymerase allows the number of nucleotides that are added to a primer being extended to be equal to or greater than 80, 90, 95, 100, 110, 120, 130, 140, 150, 175, or 200% of the number of nucleotides between the 3' end of the primer binding site and the 5'end of the next downstream primer binding site on the same strand.

[0410] In some embodiments, a DNA polymerase is used produce DNA amplicons using DNA as a template. In some embodiments, a RNA polymerase is used produce RNA amplicons using DNA as a template. In some embodiments, a reverse transcriptase is used produce cDNA amplicons using RNA as a template.

[0411] In some embodiments, the low level of 5´→ 3´ exonuclease of the polymerase is less than 80, 70, 60, 50, 40, 30, 20, 10, 5, 1, or 0.1% of the activity of the same amount of *Thermus aquaticus* polymerase ("Taq" polymerase, which is a commonly used DNA polymerase from a thermophilic bacterium, PDB 1BGX, EC 2.7.7.7, Murali *et al*., "Crystal structure of Taq DNA

polymerase in complex with an inhibitory Fab: the Fab is directed against an intermediate in the helix-coil dynamics of the enzyme," Proc. Natl. Acad. Sci. USA 95:12562-12567, 1998, which is hereby incorporated by reference in its entirety) under the same conditions. In some embodiments, the low level of strand displacement activity of the polymerase is less than 80, 70, 60, 50, 40, 30, 20, 10, 5, 1, or 0.1% of the activity of the same amount of Taq polymerase under the same conditions.

[0412] In some embodiments, the polymerase is a PUSHION DNA polymerase, such as PHUSION High Fidelity DNA polymerase (M0530S, New England BioLabs, Inc.) or PHUSION Hot Start Flex DNA polymerase (M0535S, New England BioLabs, Inc.; Frey and Suppman *BioChemica*. 2:34-35, 1995; Chester and Marshak *Analytical Biochemistry*. 209:284-290, 1993, which are each hereby incorporated by reference in its entirety). The PHUSION DNA polymerase is a *Pyrococcus*-like enzyme fused with a processivity-enhancing domain. PHUSION DNA polymerase possesses 5′→ 3′ polymerase activity and 3′→ 5′ exonuclease activity, and generates blunt-ended products. PHUSION DNA polymerase lacks 5′→ 3′ exonuclease activity and strand displacement activity.

[0413] In some embodiments, the polymerase is a Q5® DNA Polymerase, such as Q5® High-Fidelity DNA Polymerase (M0491S, New England BioLabs, Inc.) or Q5® Hot Start High-Fidelity DNA Polymerase (M0493S, New England BioLabs, Inc.). Q5® High-Fidelity DNA polymerase is a high-fidelity, thermostable, DNA polymerase with 3′→ 5′ exonuclease activity, fused to a processivity-enhancing Sso7d domain. Q5® High-Fidelity DNA polymerase lacks 5′→ 3′ exonuclease activity and strand displacement activity.

[0414] In some embodiments, the polymerase is a T4 DNA polymerase (M0203S, New England BioLabs, Inc.; Tabor and Struh. (1989). "DNA-Dependent DNA Polymerases," In Ausebel et al. (Ed.), *Current Protocols in Molecular Biology*. 3.5.10-3.5.12. New York: John Wiley & Sons, Inc., 1989; Sambrook et al. *Molecular Cloning: A Laboratory Manual*. (2nd ed.), 5.44-5.47. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989, which are each hereby incorporated by reference in its entirety). T4 DNA Polymerase catalyzes the synthesis of DNA in the 5′→ 3′ direction and requires the presence of template and primer. This enzyme has a 3′→ 5′ exonuclease activity which is much more active than that found in DNA Polymerase I. T4 DNA polymerase lacks 5′→ 3′ exonuclease activity and strand displacement activity.

[0415] In some embodiments, the polymerase is a *Sulfolobus* DNA Polymerase IV (M0327S, New England BioLabs, Inc.; (Boudsocq, *et al.* (2001). *Nucleic Acids Res.,* 29:4607-4616, 2001;

McDonald, *et al.* (2006). *Nucleic Acids Res.,* 34:1102-1111, 2006, which are each hereby incorporated by reference in its entirety). *Sulfolobus* DNA Polymerase IV is a thermostable Y-family lesion-bypass DNA Polymerase that efficiently synthesizes DNA across a variety of DNA template lesions McDonald, J.P. et al. (2006). *Nucleic Acids Res.,*. 34, 1102-1111, which is hereby incorporated by reference in its entirety). *Sulfolobus* DNA Polymerase IV lacks 5′→ 3′ exonuclease activity and strand displacement activity.

[0416] In some embodiments, if a primer binds a region with a SNP, the primer may bind and amplify the different alleles with different efficiencies or may only bind and amplify one allele. For subjects who are heterozygous, one of the alleles may not be amplified by the primer. In some embodiments, a primer is designed for each allele. For example, if there are two alleles (*e.g.*, a biallelic SNP), then two primers can be used to bind the same location of a target locus (*e.g.*, a forward primer to bind the "A" allele and a forward primer to bind the "B" allele). Standard methods, such as the dbSNP database, can be used to determine the location of known SNPs, such as SNP hot spots that have a high heterozygosity rate.

[0417] In some embodiments, the amplicons are similar in size. In some embodiments, the range of the length of the target amplicons is less than 100, 75, 50, 25, 15, 10, or 5 nucleotides. In some embodiments (such as the amplification of target loci in fragmented DNA or RNA), the length of the target amplicons is between 50 and 100 nucleotides, such as between 60 and 80 nucleotides, or 60 and 75 nucleotides, inclusive. In some embodiments (such as the amplification of multiple target loci throughout an exon or gene), the length of the target amplicons is between 100 and 500 nucleotides, such as between 150 and 450 nucleotides, 200 and 400 nucleotides, 200 and 300 nucleotides, or 300 and 400 nucleotides, inclusive.

[0418] In some embodiments, multiple target loci are simultaneously amplified using a primer pair that includes a forward and reverse primer for each target locus to be amplified in that reaction volume. In some embodiments, one round of PCR is performed with a single primer per target locus, and then a second round of PCR is performed with a primer pair per target locus. For example, the first round of PCR may be performed with a single primer per target locus such that all the primers bind the same strand (such as using a forward primer for each target locus). This allows the PCR to amplify in a linear manner and reduces or eliminates amplification bias between amplicons due to sequence or length differences. In some embodiments, the amplicons are then amplified using a forward and reverse primer for each target locus.

Exemplary Whole Genome Amplification Methods

[0419] In some embodiments, a method of the present disclosure may involve amplifying DNA, such as the use of whole genome application to amplify a nucleic acid sample before amplifying just the target loci. Amplification of the DNA, a process which transforms a small amount of genetic material to a larger amount of genetic material that comprises a similar set of genetic data, can be done by a wide variety of methods, including, but not limited to polymerase chain reaction (PCR). One method of amplifying DNA is whole genome amplification (WGA). There are a number of methods available for WGA: ligation-mediated PCR (LM-PCR), degenerate oligonucleotide primer PCR (DOP-PCR), and multiple displacement amplification (MDA). In LM-PCR, short DNA sequences called adapters are ligated to blunt ends of DNA. These adapters contain universal amplification sequences, which are used to amplify the DNA by PCR. In DOP-PCR, random primers that also contain universal amplification sequences are used in a first round of annealing and PCR. Then, a second round of PCR is used to amplify the sequences further with the universal primer sequences. MDA uses the phi-29 polymerase, which is a highly processive and non-specific enzyme that replicates DNA and has been used for single-cell analysis. The major limitations to amplification of material from a single cell are (1) necessity of using extremely dilute DNA concentrations or extremely small volume of reaction mixture, and (2) difficulty of reliably dissociating DNA from proteins across the whole genome. Regardless, single-cell whole genome amplification has been used successfully for a variety of applications for a number of years. There are other methods of amplifying DNA from a sample of DNA. The DNA amplification transforms the initial sample of DNA into a sample of DNA that is similar in the set of sequences, but of much greater quantity. In some cases, amplification may not be required.

[0420] In some embodiments, DNA may be amplified using a universal amplification, such as WGA or MDA. In some embodiments, DNA may be amplified by targeted amplification, for example using targeted PCR, or circularizing probes. In some embodiments, the DNA may be preferentially enriched using a targeted amplification method, or a method that results in the full or partial separation of desired from undesired DNA, such as capture by hybridization approaches. In some embodiments, DNA may be amplified by using a combination of a universal amplification method and a preferential enrichment method. A fuller description of some of these methods can be found elsewhere in this document.

Exemplary Enrichment and Sequencing Methods

[0421] In an embodiment, a method disclosed herein uses selective enrichment techniques that preserve the relative allele frequencies that are present in the original sample of DNA at each target loci (*e.g.*, each polymorphic locus) from a set of target loci (*e.g.*, polymorphic loci). While enrichment is particularly advantageous for methods for analyzing polymorphic loci, these enrichment methods can be readily adapted for nonpolymorphic loci if desired. In some embodiments the amplification and/or selective enrichment technique may involve PCR such as ligation mediated PCR, fragment capture by hybridization, Molecular Inversion Probes, or other circularizing probes. In some embodiments, methods for amplification or selective enrichment may involve using probes where, upon correct hybridization to the target sequence, the 3-prime end or 5-prime end of a nucleotide probe is separated from the polymorphic site of the allele by a small number of nucleotides. This separation reduces preferential amplification of one allele, termed allele bias. This is an improvement over methods that involve using probes where the 3-prime end or 5-prime end of a correctly hybridized probe are directly adjacent to or very near to the polymorphic site of an allele. In an embodiment, probes in which the hybridizing region may or certainly contains a polymorphic site are excluded. Polymorphic sites at the site of hybridization can cause unequal hybridization or inhibit hybridization altogether in some alleles, resulting in preferential amplification of certain alleles. These embodiments are improvements over other methods that involve targeted amplification and/or selective enrichment in that they better preserve the original allele frequencies of the sample at each polymorphic locus, whether the sample is pure genomic sample from a single individual or mixture of individuals.

[0422] The use of a technique to enrich a sample of DNA at a set of target loci followed by sequencing as part of a method for non-invasive prenatal allele calling or ploidy calling may confer a number of unexpected advantages. In some embodiments of the present disclosure, the method involves measuring genetic data for use with an informatics based method, such as PARENTAL SUPPORT™ (PS). The ultimate outcome of some of the embodiments is the actionable genetic data of an embryo or a fetus. There are many methods that may be used to measure the genetic data of the individual and/or the related individuals as part of embodied methods. In an embodiment, a method for enriching the concentration of a set of targeted alleles is disclosed herein, the method comprising one or more of the following steps: targeted amplification of genetic material, addition of loci specific oligonucleotide probes, ligation of

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specified DNA strands, isolation of sets of desired DNA, removal of unwanted components of a reaction, detection of certain sequences of DNA by hybridization, and detection of the sequence of one or a plurality of strands of DNA by DNA sequencing methods. In some cases the DNA strands may refer to target genetic material, in some cases they may refer to primers, in some cases they may refer to synthesized sequences, or combinations thereof. These steps may be carried out in a number of different orders.

[0423] For example, a universal amplification step of the DNA prior to targeted amplification may confer several advantages, such as removing the risk of bottlenecking and reducing allelic bias. The DNA may be mixed an oligonucleotide probe that can hybridize with two neighboring regions of the target sequence, one on either side. After hybridization, the ends of the probe may be connected by adding a polymerase, a means for ligation, and any necessary reagents to allow the circularization of the probe. After circularization, an exonuclease may be added to digest to non-circularized genetic material, followed by detection of the circularized probe. The DNA may be mixed with PCR primers that can hybridize with two neighboring regions of the target sequence, one on either side. After hybridization, the ends of the probe may be connected by adding a polymerase, a means for ligation, and any necessary reagents to complete PCR amplification. Amplified or unamplified DNA may be targeted by hybrid capture probes that target a set of loci; after hybridization, the probe may be localized and separated from the mixture to provide a mixture of DNA that is enriched in target sequences.

[0424] The use of a method to target certain loci followed by sequencing as part of a method for allele calling or ploidy calling may confer a number of unexpected advantages. Some methods by which DNA may be targeted, or preferentially enriched, include using circularizing probes, linked inverted probes (LIPs, MIPs), capture by hybridization methods such as SURESELECT, and targeted PCR or ligation-mediated PCR amplification strategies.

[0425] In some embodiments, a method of the present disclosure involves measuring genetic data for use with an informatics based method, such as PARENTAL SUPPORT™ (PS), which is described further herein. PARENTAL SUPPORT™ is an informatics based approach to manipulating genetic data, aspects of which are described herein. The ultimate outcome of some of the embodiments is the actionable genetic data of an embryo or a fetus followed by a clinical decision based on the actionable data. The algorithms behind the PS method take the measured genetic data of the target individual, often an embryo or fetus, and the measured genetic data from related individuals, and are able to increase the accuracy with which the genetic state of

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the target individual is known. In an embodiment, the measured genetic data is used in the context of making ploidy determinations during prenatal genetic diagnosis. In an embodiment, the measured genetic data is used in the context of making ploidy determinations or allele calls on embryos during in vitro fertilization. There are many methods that may be used to measure the genetic data of the individual and/or the related individuals in the aforementioned contexts. The different methods comprise a number of steps, those steps often involving amplification of genetic material, addition of oligonucleotide probes, ligation of specified DNA strands, isolation of sets of desired DNA, removal of unwanted components of a reaction, detection of certain sequences of DNA by hybridization, detection of the sequence of one or a plurality of strands of DNA by DNA sequencing methods. In some cases the DNA strands may refer to target genetic material, in some cases they may refer to primers, in some cases they may refer to synthesized sequences, or combinations thereof. These steps may be carried out in a number of different orders.

[0426] Note that in theory it is possible to target any number loci in the genome, anywhere from one loci to well over one million loci. If a sample of DNA is subjected to targeting, and then sequenced, the percentage of the alleles that are read by the sequencer will be enriched with respect to their natural abundance in the sample. The degree of enrichment can be anywhere from one percent (or even less) to ten-fold, a hundred-fold, a thousand-fold or even many million-fold. In the human genome there are roughly 3 billion base pairs, and nucleotides, comprising approximately 75 million polymorphic loci. The more loci that are targeted, the smaller the degree of enrichment is possible. The fewer the number of loci that are targeted, the greater degree of enrichment is possible, and the greater depth of read may be achieved at those loci for a given number of sequence reads.

[0427] In an embodiment of the present disclosure, the targeting or preferential may focus entirely on SNPs. In an embodiment, the targeting or preferential may focus on any polymorphic site. A number of commercial targeting products are available to enrich exons. Surprisingly, targeting exclusively SNPs, or exclusively polymorphic loci, is particularly advantageous when using a method for NPD that relies on allele distributions. There are also published methods for NPD using sequencing, for example U.S. Patent 7,888,017, involving a read count analysis where the read counting focuses on counting the number of reads that map to a given chromosome, where the analyzed sequence reads do not focused on regions of the genome that

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are polymorphic. Those types of methodology that do not focus on polymorphic alleles would not benefit as much from targeting or preferential enrichment of a set of alleles.

[0428] In an embodiment of the present disclosure, it is possible to use a targeting method that focuses on SNPs to enrich a genetic sample in polymorphic regions of the genome. In an embodiment, it is possible to focus on a small number of SNPs, for example between 1 and 100 SNPs, or a larger number, for example, between 100 and 1,000, between 1,000 and 10,000, between 10,000 and 100,000 or more than 100,000 SNPs. In an embodiment, it is possible to focus on one or a small number of chromosomes that are correlated with live trisomic births, for example chromosomes 13, 18, 21, X and Y, or some combination thereof. In an embodiment, it is possible to enrich the targeted SNPs by a small factor, for example between 1.01 fold and 100 fold, or by a larger factor, for example between 100 fold and 1,000,000 fold, or even by more than 1,000,000 fold. In an embodiment of the present disclosure, it is possible to use a targeting method to create a sample of DNA that is preferentially enriched in polymorphic regions of the genome. In an embodiment, it is possible to use this method to create a mixture of DNA with any of these characteristics where the mixture of DNA contains maternal DNA and also free floating fetal DNA. In an embodiment, it is possible to use this method to create a mixture of DNA that has any combination of these factors. For example, the method described herein may be used to produce a mixture of DNA that comprises maternal DNA and fetal DNA, and that is preferentially enriched in DNA that corresponds to 200 SNPs, all of which are located on either chromosome 18 or 21, and which are enriched an average of 1000 fold. In another example, it is possible to use the method to create a mixture of DNA that is preferentially enriched in 10,000 SNPs that are all or mostly located on chromosomes 13, 18, 21, X and Y, and the average enrichment per loci is greater than 500 fold. Any of the targeting methods described herein can be used to create mixtures of DNA that are preferentially enriched in certain loci.

[0429] In some embodiments, a method of the present disclosure further includes measuring the DNA in the mixed fraction using a high throughput DNA sequencer, where the DNA in the mixed fraction contains a disproportionate number of sequences from one or more chromosomes, wherein the one or more chromosomes are taken from the group comprising chromosome 13, chromosome 18, chromosome 21, chromosome X, chromosome Y and combinations thereof.

[0430] Described herein are three methods: multiplex PCR, targeted capture by hybridization, and linked inverted probes (LIPs), which may be used to obtain and analyze measurements from

a sufficient number of polymorphic loci from a maternal plasma sample in order to detect fetal aneuploidy; this is not meant to exclude other methods of selective enrichment of targeted loci. Other methods may equally well be used without changing the essence of the method. In each case the polymorphism assayed may include single nucleotide polymorphisms (SNPs), small indels, or STRs. A preferred method involves the use of SNPs. Each approach produces allele frequency data; allele frequency data for each targeted locus and/or the joint allele frequency distributions from these loci may be analyzed to determine the ploidy of the fetus. Each approach has its own considerations due to the limited source material and the fact that maternal plasma consists of mixture of maternal and fetal DNA. This method may be combined with other approaches to provide a more accurate determination. In an embodiment, this method may be combined with a sequence counting approach such as that described in US Patent 7,888,017. The approaches described could also be used to detect fetal paternity noninvasively from maternal plasma samples. In addition each approach may be applied to other mixtures of DNA or pure DNA samples to detect the presence or absence of aneuploid chromosomes, to genotype a large number of SNP from degraded DNA samples, to detect segmental copy number variations (CNVs), to detect other genotypic states of interest, or some combination thereof.

Accurately Measuring the Allelic Distributions in a Sample

[0431] Current sequencing approaches can be used to estimate the distribution of alleles in a sample. One such method involves randomly sampling sequences from a pool DNA, termed shotgun sequencing. The proportion of a particular allele in the sequencing data is typically very low and can be determined by simple statistics. The human genome contains approximately 3 billion base pairs. So, if the sequencing method used make 100 bp reads, a particular allele will be measured about once in every 30 million sequence reads.

[0432] In an embodiment, a method of the present disclosure is used to determine the presence or absence of two or more different haplotypes that contain the same set of loci in a sample of DNA from the measured allele distributions of loci from that chromosome. The different haplotypes could represent two different homologous chromosomes from one individual, three different homologous chromosomes from a trisomic individual, three different homologous haplotypes from a mother and a fetus where one of the haplotypes is shared between the mother and the fetus, three or four haplotypes from a mother and fetus where one or two of the

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haplotypes are shared between the mother and the fetus, or other combinations. Alleles that are polymorphic between the haplotypes tend to be more informative, however any alleles where the mother and father are not both homozygous for the same allele will yield useful information through measured allele distributions beyond the information that is available from simple read count analysis.

[0433] Shotgun sequencing of such a sample, however, is extremely inefficient as it results in many sequences for regions that are not polymorphic between the different haplotypes in the sample, or are for chromosomes that are not of interest, and therefore reveal no information about the proportion of the target haplotypes. Described herein are methods that specifically target and/or preferentially enrich segments of DNA in the sample that are more likely to be polymorphic in the genome to increase the yield of allelic information obtained by sequencing. Note that for the measured allele distributions in an enriched sample to be truly representative of the actual amounts present in the target individual, it is critical that there is little or no preferential enrichment of one allele as compared to the other allele at a given loci in the targeted segments. Current methods known in the art to target polymorphic alleles are designed to ensure that at least some of any alleles present are detected. However, these methods were not designed for the purpose of measuring the unbiased allelic distributions of polymorphic alleles present in the original mixture. It is non-obvious that any particular method of target enrichment would be able to produce an enriched sample wherein the measured allele distributions would accurately represent the allele distributions present in the original unamplified sample better than any other method. While many enrichment methods may be expected, in theory, to accomplish such an aim, an ordinary person skilled in the art is well aware that there is a great deal of stochastic or deterministic bias in current amplification, targeting and other preferential enrichment methods. One embodiment of a method described herein allows a plurality of alleles found in a mixture of DNA that correspond to a given locus in the genome to be amplified, or preferentially enriched in a way that the degree of enrichment of each of the alleles is nearly the same. Another way to say this is that the method allows the relative quantity of the alleles present in the mixture as a whole to be increased, while the ratio between the alleles that correspond to each locus remains essentially the same as they were in the original mixture of DNA. For some reported methods, preferential enrichment of loci can result in allelic biases of more than 1%, more than 2%, more than 5% and even more than 10%. This preferential enrichment may be due to capture bias when using a capture by hybridization approach, or amplification bias which may be small for each

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cycle, but can become large when compounded over 20, 30 or 40 cycles. For the purposes of this disclosure, for the ratio to remain essentially the same means that the ratio of the alleles in the original mixture divided by the ratio of the alleles in the resulting mixture is between 0.95 and 1.05, between 0.98 and 1.02, between 0.99 and 1.01, between 0.995 and 1.005, between 0.998 and 1.002, between 0.999 and 1.001, or between 0.9999 and 1.0001. Note that the calculation of the allele ratios presented here may not be used in the determination of the ploidy state of the target individual, and may only a metric to be used to measure allelic bias.

[0434] In an embodiment, once a mixture has been preferentially enriched at the set of target loci, it may be sequenced using any one of the previous, current, or next generation of sequencing instruments that sequences a clonal sample (a sample generated from a single molecule; examples include ILLUMINA GAIIx, ILLUMINA HISEQ, LIFE TECHNOLOGIES SOLiD, 5500XL). The ratios can be evaluated by sequencing through the specific alleles within the targeted region. These sequencing reads can be analyzed and counted according the allele type and the rations of different alleles determined accordingly. For variations that are one to a few bases in length, detection of the alleles will be performed by sequencing and it is essential that the sequencing read span the allele in question in order to evaluate the allelic composition of that captured molecule. The total number of captured molecules assayed for the genotype can be increased by increasing the length of the sequencing read. Full sequencing of all molecules would guarantee collection of the maximum amount of data available in the enriched pool. However, sequencing is currently expensive, and a method that can measure allele distributions using a lower number of sequence reads will have great value. In addition, there are technical limitations to the maximum possible length of read as well as accuracy limitations as read lengths increase. The alleles of greatest utility will be of one to a few bases in length, but theoretically any allele shorter than the length of the sequencing read can be used. While allele variations come in all types, the examples provided herein focus on SNPs or variants contained of just a few neighboring base pairs. Larger variants such as segmental copy number variants can be detected by aggregations of these smaller variations in many cases as whole collections of SNP internal to the segment are duplicated. Variants larger than a few bases, such as STRs require special consideration and some targeting approaches work while others will not.

[0435] There are multiple targeting approaches that can be used to specifically isolate and enrich a one or a plurality of variant positions in the genome. Typically, these rely on taking advantage of the invariant sequence flanking the variant sequence. There are reports by others related to

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targeting in the context of sequencing where the substrate is maternal plasma (see, *e.g.*, Liao et al., Clin. Chem. 2011; 57(1): pp. 92-101). However, these approaches use targeting probes that target exons, and do not focus on targeting polymorphic regions of the genome. In an embodiment, a method of the present disclosure involves using targeting probes that focus exclusively or almost exclusively on polymorphic regions. In an embodiment, a method of the present disclosure involves using targeting probes that focus exclusively or almost exclusively on SNPs. In some embodiments of the present disclosure, the targeted polymorphic sites consist of at least 10% SNPs, at least 20% SNPs, at least 30% SNPs, at least 40% SNPs, at least 50% SNPs, at least 60% SNPs, at least 70% SNPs, at least 80% SNPs, at least 90% SNPs, at least 95% SNPs, at least 98% SNPs, at least 99% SNPs, at least 99.9% SNPs, or exclusively SNPs.

[0436] In an embodiment, a method of the present disclosure can be used to determine genotypes (base composition of the DNA at specific loci) and relative proportions of those genotypes from a mixture of DNA molecules, where those DNA molecules may have originated from one or a number of genetically distinct individuals. In an embodiment, a method of the present disclosure can be used to determine the genotypes at a set of polymorphic loci, and the relative ratios of the amount of different alleles present at those loci. In an embodiment the polymorphic loci may consist entirely of SNPs. In an embodiment, the polymorphic loci can comprise SNPs, single tandem repeats, and other polymorphisms. In an embodiment, a method of the present disclosure can be used to determine the relative distributions of alleles at a set of polymorphic loci in a mixture of DNA, where the mixture of DNA comprises DNA that originates from a mother, and DNA that originates from a fetus. In an embodiment, the joint allele distributions can be determined on a mixture of DNA isolated from blood from a pregnant woman. In an embodiment, the allele distributions at a set of loci can be used to determine the ploidy state of one or more chromosomes on a gestating fetus.

[0437] In an embodiment, the mixture of DNA molecules could be derived from DNA extracted from multiple cells of one individual. In an embodiment, the original collection of cells from which the DNA is derived may comprise a mixture of diploid or haploid cells of the same or of different genotypes, if that individual is mosaic (germline or somatic). In an embodiment, the mixture of DNA molecules could also be derived from DNA extracted from single cells. In an embodiment, the mixture of DNA molecules could also be derived from DNA extracted from mixture of two or more cells of the same individual, or of different individuals. In an embodiment, the mixture of DNA molecules could be derived from DNA isolated from

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biological material that has already liberated from cells such as blood plasma, which is known to contain cell free DNA. In an embodiment, the this biological material may be a mixture of DNA from one or more individuals, as is the case during pregnancy where it has been shown that fetal DNA is present in the mixture. In an embodiment, the biological material could be from a mixture of cells that were found in maternal blood, where some of the cells are fetal in origin. In an embodiment, the biological material could be cells from the blood of a pregnant which have been enriched in fetal cells.

Circularizing Probes

[0438] Some embodiments of the present disclosure involve the use of "Linked Inverted Probes" (LIPs), which have been previously described in the literature, to amplify the target loci before or after amplification using primers that are not LIPs in the multiplex PCR methods of the invention. LIPs is a generic term meant to encompass technologies that involve the creation of a circular molecule of DNA, where the probes are designed to hybridize to targeted region of DNA on either side of a targeted allele, such that addition of appropriate polymerases and/or ligases, and the appropriate conditions, buffers and other reagents, will complete the complementary, inverted region of DNA across the targeted allele to create a circular loop of DNA that captures the information found in the targeted allele. LIPs may also be called pre-circularized probes, pre-circularizing probes, or circularizing probes. The LIPs probe may be a linear DNA molecule between 50 and 500 nucleotides in length, and in an embodiment between 70 and 100 nucleotides in length; in some embodiments, it may be longer or shorter than described herein. Others embodiments of the present disclosure involve different incarnations, of the LIPs technology, such as Padlock Probes and Molecular Inversion Probes (MIPs).

[0439] One method to target specific locations for sequencing is to synthesize probes in which the 3' and 5' ends of the probes anneal to target DNA at locations adjacent to and on either side of the targeted region, in an inverted manner, such that the addition of DNA polymerase and DNA ligase results in extension from the 3' end, adding bases to single stranded probe that are complementary to the target molecule (gap-fill), followed by ligation of the new 3' end to the 5' end of the original probe resulting in a circular DNA molecule that can be subsequently isolated from background DNA. The probe ends are designed to flank the targeted region of interest. One aspect of this approach is commonly called MIPS and has been used in conjunction with array

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technologies to determine the nature of the sequence filled in. One drawback to the use of MIPs in the context of measuring allele ratios is that the hybridization, circularization and amplification steps do not happed at equal rates for different alleles at the same loci. This results in measured allele ratios that are not representative of the actual allele ratios present in the original mixture.

[0440] In an embodiment, the circularizing probes are constructed such that the region of the probe that is designed to hybridize upstream of the targeted polymorphic locus and the region of the probe that is designed to hybridize downstream of the targeted polymorphic locus are covalently connected through a non-nucleic acid backbone. This backbone can be any biocompatible molecule or combination of biocompatible molecules. Some examples of possible biocompatible molecules are poly(ethylene glycol), polycarbonates, polyurethanes, polyethylenes, polypropylenes, sulfone polymers, silicone, cellulose, fluoropolymers, acrylic compounds, styrene block copolymers, and other block copolymers.

[0441] In an embodiment of the present disclosure, this approach has been modified to be easily amenable to sequencing as a means of interrogating the filled in sequence. In order to retain the original allelic proportions of the original sample at least one key consideration must be taken into account. The variable positions among different alleles in the gap-fill region must not be too close to the probe binding sites as there can be initiation bias by the DNA polymerase resulting in differential of the variants. Another consideration is that additional variations may be present in the probe binding sites that are correlated to the variants in the gap-fill region which can result unequal amplification from different alleles. In an embodiment of the present disclosure, the 3' ends and 5' ends of the pre-circularized probe are designed to hybridize to bases that are one or a few positions away from the variant positions (polymorphic sites) of the targeted allele. The number of bases between the polymorphic site (SNP or otherwise) and the base to which the 3' end and/or 5' of the pre-circularized probe is designed to hybridize may be one base, it may be two bases, it may be three bases, it may be four bases, it may be five bases, it may be six bases, it may be seven to ten bases, it may be eleven to fifteen bases, or it may be sixteen to twenty bases, twenty to thirty bases, or thirty to sixty bases. The forward and reverse primers may be designed to hybridize a different number of bases away from the polymorphic site. Circularizing probes can be generated in large numbers with current DNA synthesis technology allowing very large numbers of probes to be generated and potentially pooled, enabling interrogation of many loci simultaneously. It has been reported to work with more than

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300,000 probes. Two papers that discuss a method involving circularizing probes that can be used to measure the genomic data of the target individual include: Porreca et al., Nature Methods, 2007 4(11), pp. 931-936.; and also Turner et al., Nature Methods, 2009, 6(5), pp. 315-316. The methods described in these papers may be used in combination with other methods described herein. Certain steps of the method from these two papers may be used in combination with other steps from other methods described herein.

[0442] In some embodiments of the methods disclosed herein, the genetic material of the target individual is optionally amplified, followed by hybridization of the pre-circularized probes, performing a gap fill to fill in the bases between the two ends of the hybridized probes, ligating the two ends to form a circularized probe, and amplifying the circularized probe, using, for example, rolling circle amplification. Once the desired target allelic genetic information is captured by circularizing appropriately designed oligonucleotide probes, such as in the LIPs system, the genetic sequence of the circularized probes may be being measured to give the desired sequence data. In an embodiment, the appropriately designed oligonucleotides probes may be circularized directly on unamplified genetic material of the target individual, and amplified afterwards. Note that a number of amplification procedures may be used to amplify the original genetic material, or the circularized LIPs, including rolling circle amplification, MDA, or other amplification protocols. Different methods may be used to measure the genetic information on the target genome, for example using high throughput sequencing, Sanger sequencing, other sequencing methods, capture-by-hybridization, capture-by-circularization, multiplex PCR, other hybridization methods, and combinations thereof.

[0443] Once the genetic material of the individual has been measured using one or a combination of the above methods, an informatics based method, such as the PARENTAL SUPPORT™ method, along with the appropriate genetic measurements, can then be used to determination the ploidy state of one or more chromosomes on the individual, and/or the genetic state of one or a set of alleles, specifically those alleles that are correlated with a disease or genetic state of interest. Note that the use of LIPs has been reported for multiplexed capture of genetic sequences, followed by genotyping with sequencing. However, the use of sequencing data resulting from a LIPs-based strategy for the amplification of the genetic material found in a single cell, a small number of cells, or extracellular DNA, has not been used for the purpose of determining the ploidy state of a target individual.

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[0444] Applying an informatics based method to determine the ploidy state of an individual from genetic data as measured by hybridization arrays, such as the ILLUMINA INFINIUM array, or the AFFYMETRIX gene chip has been described in documents references elsewhere in this document. However, the method described herein shows improvements over methods described previously in the literature. For example, the LIPs based approach followed by high throughput sequencing unexpectedly provides better genotypic data due to the approach having better capacity for multiplexing, better capture specificity, better uniformity, and low allelic bias. Greater multiplexing allows more alleles to be targeted, giving more accurate results. Better uniformity results in more of the targeted alleles being measured, giving more accurate results. Lower rates of allelic bias result in lower rates of miscalls, giving more accurate results. More accurate results result in an improvement in clinical outcomes, and better medical care.

[0445] It is important to note that LIPs may be used as a method for targeting specific loci in a sample of DNA for genotyping by methods other than sequencing. For example, LIPs may be used to target DNA for genotyping using SNP arrays or other DNA or RNA based microarrays.

Ligation-mediated PCR

[0446] Ligation-mediated PCR may be used to amplify the target loci before or after PCR amplification using primers that are not ligated. Ligation-mediated PCR is a method of PCR used to preferentially enrich a sample of DNA by amplifying one or a plurality of loci in a mixture of DNA, the method comprising: obtaining a set of primer pairs, where each primer in the pair contains a target specific sequence and a non-target sequence, where the target specific sequence is preferably designed to anneal to a target region, one upstream and one downstream from the polymorphic site, and which can be separated from the polymorphic site by 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-20, 21-30, 31-40, 41-50, 51-100, or more than 100; polymerization of the DNA from the 3-prime end of upstream primer to the fill the single strand region between it and the 5-prime end of the downstream primer with nucleotides complementary to the target molecule; ligation of the last polymerized base of the upstream primer to the adjacent 5-prime base of the downstream primer; and amplification of only polymerized and ligated molecules using the non-target sequences contained at the 5-prime end of the upstream primer and the 3-prime end of the downstream primer. Pairs of primers to distinct targets may be mixed in the same reaction. The non-target sequences serve as universal sequences such that of all pairs of

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primers that have been successfully polymerized and ligated may be amplified with a single pair of amplification primers.

Capture by Hybridization

[0447] In some embodiments, a method of the present disclosure may involve using any of the following capture by hybridization methods in addition to using multiplex PCR to amplify the target loci. Preferential enrichment of a specific set of sequences in a target genome can be accomplished in a number of ways. Elsewhere in this document is a description of how LIPs can be used to target a specific set of sequences, but in all of those applications, other targeting and/or preferential enrichment methods can be used equally well for the same ends. One example of another targeting method is the capture by hybridization approach. Some examples of commercial capture by hybridization technologies include AGILENT's SURE SELECT and ILLUMINA's TRUSEQ. In capture by hybridization, a set of oligonucleotides that is complimentary or mostly complimentary to the desired targeted sequences is allowed to hybridize to a mixture of DNA, and then physically separated from the mixture. Once the desired sequences have hybridized to the targeting oligonucleotides, the effect of physically removing the targeting oligonucleotides is to also remove the targeted sequences. Once the hybridized oligos are removed, they can be heated to above their melting temperature and they can be amplified. Some ways to physically remove the targeting oligonucleotides is by covalently bonding the targeting oligos to a solid support, for example a magnetic bead, or a chip. Another way to physically remove the targeting oligonucleotides is by covalently bonding them to a molecular moiety with a strong affinity for another molecular moiety. An example of such a molecular pair is biotin and streptavidin, such as is used in SURE SELECT. Thus that targeted sequences could be covalently attached to a biotin molecule, and after hybridization, a solid support with streptavidin affixed can be used to pull down the biotinylated oligonucleotides, to which are hybridized to the targeted sequences.

[0448] Hybrid capture involves hybridizing probes that are complementary to the targets of interest to the target molecules. Hybrid capture probes were originally developed to target and enrich large fractions of the genome with relative uniformity between targets. In that application, it was important that all targets be amplified with enough uniformity that all regions could be detected by sequencing, however, no regard was paid to retaining the proportion of alleles in

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original sample. Following capture, the alleles present in the sample can be determined by direct sequencing of the captured molecules. These sequencing reads can be analyzed and counted according the allele type. However, using the current technology, the measured allele distributions the captured sequences are typically not representative of the original allele distributions.

[0449] In an embodiment, detection of the alleles is performed by sequencing. In order to capture the allele identity at the polymorphic site, it is essential that the sequencing read span the allele in question in order to evaluate the allelic composition of that captured molecule. Since the capture molecules are often of variable lengths upon sequencing cannot be guaranteed to overlap the variant positions unless the entire molecule is sequenced. However, cost considerations as well as technical limitations as to the maximum possible length and accuracy of sequencing reads make sequencing the entire molecule unfeasible. In an embodiment, the read length can be increased from about 30 to about 50 or about 70 bases can greatly increase the number of reads that overlap the variant positions within the targeted sequences.

[0450] Another way to increase the number of reads that interrogate the position of interest is to decrease the length of the probe, as long as it does not result in bias in the underlying enriched alleles. The length of the synthesized probe should be long enough such that two probes designed to hybridize to two different alleles found at one locus will hybridize with near equal affinity to the various alleles in the original sample. Currently, methods known in the art describe probes that are typically longer than 120 bases. In a current embodiment, if the allele is one or a few bases then the capture probes may be less than about 110 bases, less than about 100 bases, less than about 90 bases, less than about 80 bases, less than about 70 bases, less than about 60 bases, less than about 50 bases, less than about 40 bases, less than about 30 bases, and less than about 25 bases, and this is sufficient to ensure equal enrichment from all alleles. When the mixture of DNA that is to be enriched using the hybrid capture technology is a mixture comprising free floating DNA isolated from blood, for example maternal blood, the average length of DNA is quite short, typically less than 200 bases. The use of shorter probes results in a greater chance that the hybrid capture probes will capture desired DNA fragments. Larger variations may require longer probes. In an embodiment, the variations of interest are one (a SNP) to a few bases in length. In an embodiment, targeted regions in the genome can be preferentially enriched using hybrid capture probes wherein the hybrid capture probes are of a length below 90 bases, and can be less than 80 bases, less than 70 bases, less than 60 bases, less than 50 bases, less than

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40 bases, less than 30 bases, or less than 25 bases. In an embodiment, to increase the chance that the desired allele is sequenced, the length of the probe that is designed to hybridize to the regions flanking the polymorphic allele location can be decreased from above 90 bases, to about 80 bases, or to about 70 bases, or to about 60 bases, or to about 50 bases, or to about 40 bases, or to about 30 bases, or to about 25 bases.

[0451] There is a minimum overlap between the synthesized probe and the target molecule in order to enable capture. This synthesized probe can be made as short as possible while still being larger than this minimum required overlap. The effect of using a shorter probe length to target a polymorphic region is that there will be more molecules that overlap the target allele region. The state of fragmentation of the original DNA molecules also affects the number of reads that will overlap the targeted alleles. Some DNA samples such as plasma samples are already fragmented due to biological processes that take place in vivo. However, samples with longer fragments by benefit from fragmentation prior to sequencing library preparation and enrichment. When both probes and fragments are short (~60-80 bp) maximum specificity may be achieved relatively few sequence reads failing to overlap the critical region of interest.

[0452] In an embodiment, the hybridization conditions can be adjusted to maximize uniformity in the capture of different alleles present in the original sample. In an embodiment, hybridization temperatures are decreased to minimize differences in hybridization bias between alleles. Methods known in the art avoid using lower temperatures for hybridization because lowering the temperature has the effect of increasing hybridization of probes to unintended targets. However, when the goal is to preserve allele ratios with maximum fidelity, the approach of using lower hybridization temperatures provides optimally accurate allele ratios, despite the fact that the current art teaches away from this approach. Hybridization temperature can also be increased to require greater overlap between the target and the synthesized probe so that only targets with substantial overlap of the targeted region are captured. In some embodiments of the present disclosure, the hybridization temperature is lowered from the normal hybridization temperature to about 40°C, to about 45°C, to about 50°C, to about 55°C, to about 60°C, to about 65, or to about 70°C.

[0453] In an embodiment, the hybrid capture probes can be designed such that the region of the capture probe with DNA that is complementary to the DNA found in regions flanking the polymorphic allele is not immediately adjacent to the polymorphic site. Instead, the capture probe can be designed such that the region of the capture probe that is designed to hybridize to

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the DNA flanking the polymorphic site of the target is separated from the portion of the capture probe that will be in van der Waals contact with the polymorphic site by a small distance that is equivalent in length to one or a small number of bases. In an embodiment, the hybrid capture probe is designed to hybridize to a region that is flanking the polymorphic allele but does not cross it; this may be termed a flanking capture probe. The length of the flanking capture probe may be less than about 120 bases, less than about 110 bases, less than about 100 bases, less than about 90 bases, and can be less than about 80 bases, less than about 70 bases, less than about 60 bases, less than about 50 bases, less than about 40 bases, less than about 30 bases, or less than about 25 bases. The region of the genome that is targeted by the flanking capture probe may be separated by the polymorphic locus by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-20, or more than 20 base pairs.

[0454] Description of a targeted capture based disease screening test using targeted sequence capture. Custom targeted sequence capture, like those currently offered by AGILENT (SURE SELECT), ROCHE-NIMBLEGEN, or ILLUMINA. Capture probes could be custom designed to ensure capture of various types of mutations. For point mutations, one or more probes that overlap the point mutation should be sufficient to capture and sequence the mutation.

[0455] For small insertions or deletions, one or more probes that overlap the mutation may be sufficient to capture and sequence fragments comprising the mutation. Hybridization may be less efficient between the probe-limiting capture efficiency, typically designed to the reference genome sequence. To ensure capture of fragments comprising the mutation one could design two probes, one matching the normal allele and one matching the mutant allele. A longer probe may enhance hybridization. Multiple overlapping probes may enhance capture. Finally, placing a probe immediately adjacent to, but not overlapping, the mutation may permit relatively similar capture efficiency of the normal and mutant alleles.

[0456] For Simple Tandem Repeats (STRs), a probe overlapping these highly variable sites is unlikely to capture the fragment well. To enhance capture a probe could be placed adjacent to, but not overlapping the variable site. The fragment could then be sequenced as normal to reveal the length and composition of the STR.

[0457] For large deletions, a series of overlapping probes, a common approach currently used in exon capture systems may work. However, with this approach it may be difficult to determine whether or not an individual is heterozygous. Targeting and evaluating SNPs within the captured region could potentially reveal loss of heterozygosity across the region indicating that an

individual is a carrier. In an embodiment, it is possible to place non-overlapping or singleton probes across the potentially deleted region and use the number of fragments captured as a measure of heterozygosity. In the case where an individual caries a large deletion, one-half the number of fragments are expected to be available for capture relative to a non-deleted (diploid) reference locus. Consequently, the number of reads obtained from the deleted regions should be roughly half that obtained from a normal diploid locus. Aggregating and averaging the sequencing read depth from multiple singleton probes across the potentially deleted region may enhance the signal and improve confidence of the diagnosis. The two approaches, targeting SNPs to identify loss of heterozygosity and using multiple singleton probes to obtain a quantitative measure of the quantity of underlying fragments from that locus can also be combined. Either or both of these strategies may be combined with other strategies to better obtain the same end.

[0458] If during testing cfDNA detection of a male fetus, as indicated by the presence of the Y-chromosome fragments, captured and sequenced in the same test, and either an X-linked dominant mutation where mother and father are unaffected, or a dominant mutation where mother is not affected would indicated heighted risk to the fetus. Detection of two mutant recessive alleles within the same gene in an unaffected mother would imply the fetus had inherited a mutant allele from father and potentially a second mutant allele from mother. In all cases, follow-up testing by amniocentesis or chorionic villus sampling may be indicated.

[0459] A targeted capture based disease screening test could be combined with a targeted capture based non-invasive prenatal diagnostic test for aneuploidy.

[0460] There are a number of ways to decrease depth of read (DOR) variability: for example, one could increase primer concentrations, one could use longer targeted amplification probes, or one could run more STA cycles (such as more than 25, more than 30, more than 35, or even more than 40)

Exemplary Methods of Determining the Number of DNA Molecules in a Sample.

[0461] A method is described herein to determine the number of DNA molecules in a sample by generating a uniquely identified molecule for each original DNA molecules in the sample during the first round of DNA amplification. Described here is a procedure to accomplish the above end followed by a single molecule or clonal sequencing method.

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[0462] The approach entails targeting one or more specific loci and generating a tagged copy of the original molecules such manner that most or all of the tagged molecules from each targeted locus will have a unique tag and can be distinguished from one another upon sequencing of this barcode using clonal or single molecule sequencing. Each unique sequenced barcode represents a unique molecule in the original sample. Simultaneously, sequencing data is used to ascertain the locus from which the molecule originates. Using this information one can determine the number of unique molecules in the original sample for each locus.

[0463] This method can be used for any application in which quantitative evaluation of the number of molecules in an original sample is required. Furthermore, the number of unique molecules of one or more targets can be related to the number of unique molecules to one or more other targets to determine the relative copy number, allele distribution, or allele ratio. Alternatively, the number of copies detected from various targets can be modeled by a distribution in order to identify the mostly likely number of copies of the original targets. Applications include but are not limited to detection of insertions and deletions such as those found in carriers of Duchenne Muscular Dystrophy; quantitation of deletions or duplications segments of chromosomes such as those observed in copy number variants; chromosome copy number of samples from born individuals; chromosome copy number of samples from unborn individuals such as embryos or fetuses.

[0464] The method can be combined with simultaneous evaluation of variations contained in the targeted by sequence. This can be used to determine the number of molecules representing each allele in the original sample. This copy number method can be combined with the evaluation of SNPs or other sequence variations to determine the chromosome copy number of born and unborn individuals; the discrimination and quantification of copies from loci which have short sequence variations, but in which PCR may amplifies from multiple target regions such as in carrier detection of Spinal Muscle Atrophy; determination of copy number of different sources of molecules from samples consisting of mixtures of different individual such as in detection of fetal aneuploidy from free floating DNA obtained from maternal plasma.

[0465] In an embodiment, the method as it pertains to a single target locus may comprise one or more of the following steps: (1) Designing a standard pair of oligomers for PCR amplification of a specific locus. (2) Adding, during synthesis, a sequence of specified bases with no or minimal complementarity to the target locus or genome to the 5' end of the one of the target specific oligomer. This sequence, termed the tail, is a known sequence, to be used for subsequent

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amplification, followed by a sequence of random nucleotides. These random nucleotides comprise the random region. The random region comprises a randomly generated sequence of nucleic acids that probabilistically differ between each probe molecule. Consequently, following synthesis, the tailed oligomer pool will consists of a collection of oligomers beginning with a known sequence followed by unknown sequence that differs between molecules, followed by the target specific sequence. (3) Performing one round of amplification (denaturation, annealing, extension) using only the tailed oligomer. (4) Adding exonuclease to the reaction, effectively stopping the PCR reaction, and incubating the reaction at the appropriate temperature to remove forward single stranded oligos that did not anneal to temple and extend to form a double stranded product. (5) Incubating the reaction at a high temperature to denature the exonuclease and eliminate its activity. (6) Adding to the reaction a new oligonucleotide that is complementary to tail of the oligomer used in the first reaction along with the other target specific oligomer to enable PCR amplification of the product generated in the first round of PCR. (7) Continuing amplification to generate enough product for downstream clonal sequencing. (8) Measuring the amplified PCR product by a multitude of methods, for example, clonal sequencing, to a sufficient number of bases to span the sequence.

[0466] In an embodiment, a method of the present disclosure involves targeting multiple loci in parallel or otherwise. Primers to different target loci can be generated independently and mixed to create multiplex PCR pools. In an embodiment, original samples can be divided into sub-pools and different loci can be targeted in each sub-pool before being recombined and sequenced. In an embodiment, the tagging step and a number of amplification cycles may be performed before the pool is subdivided to ensure efficient targeting of all targets before splitting, and improving subsequent amplification by continuing amplification using smaller sets of primers in subdivided pools.

[0467] One example of an application where this technology would be particularly useful is non-invasive prenatal aneuploidy diagnosis where the ratio of alleles at a given locus or a distribution of alleles at a number of loci can be used to help determine the number of copies of a chromosome present in a fetus. In this context, it is desirable to amplify the DNA present in the initial sample while maintaining the relative amounts of the various alleles. In some circumstances, especially in cases where there is a very small amount of DNA, for example, fewer than 5,000 copies of the genome, fewer than 1,000 copies of the genome, fewer than 500 copies of the genome, and fewer than 100 copies of the genome, one can encounter a

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phenomenon called bottlenecking. This is where there are a small number of copies of any given allele in the initial sample, and amplification biases can result in the amplified pool of DNA having significantly different ratios of those alleles than are in the initial mixture of DNA. By applying a unique or nearly unique set of barcodes to each strand of DNA before standard PCR amplification, it is possible to exclude n-1 copies of DNA from a set of n identical molecules of sequenced DNA that originated from the same original molecule.

[0468] For example, imagine a heterozygous SNP in the genome of an individual, and a mixture of DNA from the individual where ten molecules of each allele are present in the original sample of DNA. After amplification there may be 100,000 molecules of DNA corresponding to that locus. Due to stochastic processes, the ratio of DNA could be anywhere from 1:2 to 2:1, however, since each of the original molecules was tagged with a unique tag, it would be possible to determine that the DNA in the amplified pool originated from exactly 10 molecules of DNA from each allele. This method would therefore give a more accurate measure of the relative amounts of each allele than a method not using this approach. For methods where it is desirable for the relative amount of allele bias to be minimized, this method will provide more accurate data.

[0469] Association of the sequenced fragment to the target locus can be achieved in a number of ways. In an embodiment, a sequence of sufficient length is obtained from the targeted fragment to span the molecule barcode as well a sufficient number of unique bases corresponding to the target sequence to allow unambiguous identification of the target locus. In another embodiment, the molecular bar-coding primer that contains the randomly generated molecular barcode can also contain a locus specific barcode (locus barcode) that identifies the target to which it is to be associated. This locus barcode would be identical among all molecular bar-coding primers for each individual target and hence all resulting amplicons, but different from all other targets. In an embodiment, the tagging method described herein may be combined with a one-sided nesting protocol.

[0470] In an embodiment, the design and generation of molecular barcoding primers may be reduced to practice as follows: the molecular barcoding primers may consist of a sequence that is not complementary to the target sequence followed by random molecular barcode region followed by a target specific sequence. The sequence 5' of molecular barcode may be used for subsequence PCR amplification and may comprise sequences useful in the conversion of the amplicon to a library for sequencing. The random molecular barcode sequence could be

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generated in a multitude of ways. The preferred method synthesize the molecule tagging primer in such a way as to include all four bases to the reaction during synthesis of the barcode region. All or various combinations of bases may be specified using the IUPAC DNA ambiguity codes. In this manner the synthesized collection of molecules will contain a random mixture of sequences in the molecular barcode region. The length of the barcode region will determine how many primers will contain unique barcodes. The number of unique sequences is related to the length of the barcode region as N^L where N is the number of bases, typically 4, and L is the length of the barcode. A barcode of five bases can yield up to 1024 unique sequences; a barcode of eight bases can yield 65536 unique barcodes. In an embodiment, the DNA can be measured by a sequencing method, where the sequence data represents the sequence of a single molecule. This can include methods in which single molecules are sequenced directly or methods in which single molecules are amplified to form clones detectable by the sequence instrument, but that still represent single molecules, herein called clonal sequencing.

Exemplary Methods and Reagents for Quantification of Amplification Products

[0471] Quantitation of specific nucleic acid sequences of interest is typically done by quantitative real-time PCR techniques such as TAQMAN (LIFE TECHNOLOGIES), INVADER probes (THIRD WAVE TECHNOLOGIES), and the like. Such techniques suffer from numerous shortcomings such as limited ability to achieve the simultaneous analysis of multiple sequences in parallel (multiplexation) and the ability to provide accurate quantitative data for only a narrow range of possible amplification cycles (*e.g.*, when the logarithm of PCR amplification production quantity versus the number of cycles is in the linear range). DNA sequencing techniques, particularly high throughput next-generation sequencing techniques (often referred to as massively parallel sequencing techniques) such as those employed in MYSEQ (ILLUMINA), HISEQ (ILLUMINA), ION TORRENT (LIFE TECHNOLOGIES), GENOME ANALYZER ILX (ILLUMINA), GS FLEX+ (ROCHE 454) *etc.*, can be used for by quantitative measurements of the number of copies of sequence of interest present in sample, thereby providing quantitative information about the starting materials, *e.g.*, copy number or transcription levels. High throughput genetic sequencers are amenable to the use of bar coding (*i.e.*, sample tagging with distinctive nucleic acid sequences) so as to identify specific samples from individuals thereby permitting the simultaneous analysis of multiple samples in a single

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run of the DNA sequencer. The number of times a given region of the genome in a library preparation (or other nucleic preparation of interest) is sequenced (number of reads) will be proportional to the number of copies of that sequence in the genome of interest (or expression level in the case of cDNA containing preparations). However, the preparation and sequencing of genetic libraries (and similar genome derived preparations) can introduce numerous biases that interfere with obtaining an accurate quantitative reading for the nucleic acid sequence of interest. For example, different nucleic acid sequences can amplify with different efficiencies during nucleic amplification steps that take place during the genetic library preparation or sample preparation.

[0472] The problem with differential amplification efficiencies can be mitigated by using certain embodiments of the subject invention. The subject invention includes various methods and compositions that relate to the use of standards for inclusion in amplification processes that can be used to improve the accuracy of quantitation. The invention is of use in, among other areas, the detection of aneuploidy in a fetus by analyzing free floating fetal DNA in maternal blood, as described herein and as described, among other places, U.S. Patent No. 8,008,018; U.S. Patent No. 7,332,277; PCT Published Application WO 2012/078792A2; and PCT Published Application WO 2011/146632 A1, which are each herein incorporated by reference in its entirety Embodiments of the invention are also of use in the detection of aneuploidy in an *in vitro* generated embryos. Commercially significant aneuploidies that may be detected include aneuploidy of the human chromosomes 13, 18, 21, X and Y.

[0473] Embodiments of the invention may be used with either human or non-human nucleic acids, and may be applied to both animal and plant derived nucleic acids. Embodiments of the invention may also be used to detect and/or quantitate alleles for other genetic disorders characterized by deletions or insertions. The deletion containing alleles can be detected in suspected carriers of the allele of interest.

[0474] One embodiment of the subject invention includes standards that are present in a known quantity (relative or absolute). For example, consider a genetic library made from a genetic source that is diploid for chromosome 8 (containing locus A) and triploid for chromosome 21 (containing locus B). A genetic library can be produced from this sample that will contain sequences in quantities that are a function of the number of chromosomes present in the sample, *e.g.*, 200 copies of locus A and 300 copies of locus B. However, if locus A amplifies much more efficiently than locus B, after PCR there may be 60,000 copies of the A amplicon and 30,000

copies of the B amplicon, thus obscuring the true chromosomal copy number of the initial genomic sample when analysis by high throughput DNA sequencing (or other quantitative nucleic acid detection techniques). To mitigate this problem a standard sequence for locus A is employed, wherein the standard sequence amplifies with essentially the same efficiency as locus A. Similarly, a standard sequence for locus B is created, wherein the standard sequence amplifies with the essentially the same efficiency as locus B. A standard sequence of locus A and a standard sequence for locus B are added to the mixture prior to PCR (or other amplification techniques). These standard sequences are present in known quantities, either relative quantities or absolute quantities. Thus if a 1:1 mixture of standard sequence A and standard sequence B were added (prior to amplification) to the mixture in the previous example, 3000 copies of the standard A amplicon would be produced and 1000 copies of the standard B amplicon would be produced, showing that locus A is amplified 3 times more efficiently than locus B, under the same set of conditions.

[0475] In various embodiments one or more selected regions of a genome containing a SNP (or other polymorphism) of interest can be specifically amplified and subsequently sequenced. This target specific amplification can take place during the formation of a genetic library for sequencing. The library can contain numerous targeted regions for amplification. In some embodiments at least 10; 100, 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 regions of interest. Examples of such libraries are described herein and can be found in U.S. Patent Application No. 2012/0270212, filed November 18, 2011, which is herein incorporated by reference in its entirety.

[0476] Many high throughput DNA sequencing techniques require the modification of the genetic starting material, *e.g.*, the litigation of universal priming sites and/or barcodes, so as to form libraries to facilitate the clonal amplification of small nucleic acid fragments prior to performing subsequent sequencing reactions. In some embodiments, one or more standard sequences are added during genetic library formation or added to a precursor component of a genetic library prior to amplification of the library. The standard sequences can be selected so as to mimic (yet be distinguishable based on nucleotide base sequence) target genomic fragments to be prepared for sequencing by a high throughput genetic sequencing technique. In one embodiment, the standard sequence can be identical to the target genomic fragment excepting one, two, three, four to ten, or eleven to twenty nucleotides. In some embodiments, when the

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target genetic sequence contains a SNP, the standard sequence can be identical to the SNP excepting the nucleotide at the polymorphic base, which may be chosen to be one of the four nucleotides that is not observed at that location in nature. The standard sequences can be used in a highly multiplexed analysis of multiple target loci (such as polymorphic loci). Standard sequences can be added during the process of library formation (prior to amplification) in known quantities (relative or absolute) so as to provide a standard metric for greater accuracy in determining the amount of target sequence of interest in the sample of analysis. The combination of knowledge of the known quantities of the standard sequences used in conjunction with the knowledge of the ploidy level formation of library for sequencing formed from a genome of previously characterized ploidy level, *e.g.*, known to be diploid for all autosomal chromosomes, can be used to calibrate the amplification properties of each standard sequence with respect to its corresponding target sequence and account for variations between batches of mixtures comprising multiple standard sequences. Given that it is often necessary to simultaneously analyze a large number of loci, it is useful to produce a mixture comprising a large set standard sequences. Embodiments of the invention include mixtures comprising multiple standard sequences. Ideally the amount of each standard sequence in the mixture is known with high precision. However, it is extremely difficult to achieve this ideal because as a practical matter there is a significant amount of variation in the quantity of each standard sequence in the mixture, particularly for mixtures comprising a large number of different synthetic oligonucleotides. This variation has numerous sources, *e.g.*, variations in *in vitro* oligonucleotide synthesis reaction efficiencies between batch, inaccuracies in volume measurement, variations in pipetting, Furthermore, this variation can occur between different batches of that theoretically contain the exact same set of standard sequences in the exact same amounts. Accordingly, it is of interest to calibrate each batch of standard sequences independently. Batches of standard sequences can be calibrated against reference genomes of known chromosomal composition. Batched of standard sequences can be calibrated by sequencing the batch of standard sequences with minimal or no amplifications steps included in the sequencing protocol. Embodiments of the invention include calibrated mixtures of different standard sequences. Other embodiments of the invention include methods of calibrating mixtures of different standard sequences and calibrated mixtures of different standard sequences made by the subject methods.

[0477] Various embodiments of the subject mixtures of standard sequences and methods for using them can comprise at least 10; 100, 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000;

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15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 or more standards sequences, as well as various intermediate amounts. The number of the standard sequences can be the same as the number of target sequences selected for analysis during the generation of a targeted library for DNA sequencing. However, in some embodiments, it may be advantageous to use a lower number of standard sequences than the number of targeted regions in the library being constructed. It may be advantageous to use the lower number so as avoid coming up against the limits of the sequencing capacity of the high throughput DNA sequencer being employed. The number of standard sequences can be 50% or less than the number of targeted regions, 40% or less than the number of targeted regions, be 30% or less than the number of targeted regions, 20% or less than the number of targeted regions, be 10% or less than the number of targeted regions, 5% or less than the number of targeted regions, 1% or less than the number of targeted regions, as well as various intermediate values. For example, if a genetic library is created using 15,000 pairs of primers targeted to specific SNP containing loci, a suitable a mixture containing 1500 standard sequences corresponding to 1500 of the 15,000 targeted loci can be added prior to the amplification step of library constructions.

[0478] The amount of standard sequences added during library construction can vary considerably among different embodiments. In some embodiments, the amount of each standard sequence can be approximately the same as the predicted amount of the target sequence present in the genomic material sample used for library preparation. In other embodiments, the amount of each standard sequence can be greater or less than the predicted amount of the target sequence present in the genomic material sample used for library preparation. While the initial relative amounts of the target sequence and the standard sequence are not critical for the function of the invention, it is preferable that the amount be within the range 100 times greater to 100 times less than the amount of the target sequence present in the genomic material sample used for library preparation. Excessive amounts of standard may use too much sequencing capacity of the DNA sequencer in a given run of the instrument. Using too low an amount of standard sequences will produce insufficient data to aid in the analysis of variation in amplification efficiency.

[0479] The standard sequences may be selected to be very similar in nucleotide base sequence to the amplified regions of interest; preferably the standard sequence has the exact same primer-binding sites as the analyzed genomic region, *i.e.*, the "target sequence." The standard sequence must be distinguishable from the corresponding target sequence at a given locus. For the sake of convenience, this distinguishable region of the standard sequence will be referred to as a

"marker sequence." In some embodiments, the marker sequence region of the target sequences contains the polymorphic region, *e.g.*, a SNP, and can be flanked on both sides by primer binding regions. The standard sequence may be selected to closely match the GC content of the corresponding target sequence. In some embodiments, the primer binding regions of the standard sequence are flanked by universal priming sites. These universal priming sites are selected to match universal priming sites used in a genomic library for analysis. In other embodiments, the standard sequences do not have universal priming sites and the universal priming sites are added during the creation of a library. Standard sequences are typically provided in single stranded form. A standard sequence is defined with respect to a corresponding target sequence and the sequence specific reagents used to amplify the target sequence. In some embodiments, the target sequence contains the polymorphism of interest, *e.g.*, a SNP, a deletion, or insertion, present in the nucleic acid sample for analysis. The standard sequence is a synthetic polynucleotide that is similar in nucleotide base sequence to the target sequence, but is nonetheless distinguishable from the target sequence by virtue of at least one nucleotide base difference, thereby providing a mechanism for distinguishing amplicon sequences derived from the standard sequence form amplicon sequences derived from the target sequence. Standard sequences are selected so as to have essentially the same amplification properties as the corresponding target sequence when amplified with the same set of amplification reagents, *e.g.*, PCR primers. In some embodiments, the standard sequences can have the same primer sequence binding sites than the corresponding target sequences. In other embodiments, the standard sequences can have a different primer sequence binding sites than the corresponding target sequences. In some embodiments, the standard sequences can be selected to produce amplicons that have the same length as the length of amplicons produced from the corresponding target sequences. In other embodiments, the standard sequences can be selected to produce amplicons that have the slightly different lengths than the length of amplicons produced from the corresponding target sequences.

[0480] After the amplification reactions have been completed, the library is sequenced on a high throughput DNA sequencer where individual molecule are clonally amplified and sequenced. The number of sequence reads for each allele of the target sequence is counted, also counted are the number of sequence reads for the standard sequence corresponding to the target sequence. The process is also carried out for at least one other pair of target sequences and corresponding standard sequences. Consider for example, locus A, X_{A1}reads for allele 1 of locus A are

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produced; X_{A2} reads for allele 2 of locus A are produced, and X_{AC} reads for standard sequence A are produced. The ratio of (X_{A1} plus X_{A2}) to X_{AC} is determined for each locus of interest. As discussed earlier, the process can be performed on a reference genome, *e.g.*, a genome that is known to be diploid for all chromosomes. The process can be repeated many times in order to provide a large number of read values so as to determine a mean number of reads and the standard deviation in the number of reads. The process is performed with a mixture comprising a large number of different standard sequences corresponding to different loci. By assuming that (1) X_{A1} plus X_{A2} corresponds to the known number of chromosome, *e.g.*, 2 for the normal human female genome and (2) the standard sequences have similar amplification (and detectability) properties as their corresponding natural loci, the relative amounts of the different standard sequences in the multiplex standard mixture can be determined. The calibrated multiplex standard sequence mixture can then be used to adjust for the variability in amplification efficiency between the different loci in a multiplex amplification reaction.

[0481] Other embodiments of the invention include methods and compositions for measuring the copy number of specific genes of interest, including duplications and mutant genes characterized by large deletions that would interfere with quantitation by sequencing. Sequencing would have problems detecting alleles having such deletions. Standard sequences included the amplification process can be used to reduce this problem.

[0482] In one embodiment of the invention the target sequence for analysis is a gene having a wild type (*i.e.* functional) form and a mutant form characterized by a deletion. Exemplary of such genes is SMN1, an allele having deletion being responsible for the genetic disease spinal muscular atrophy (SMA). It is of interest to detect an individual carrying the mutant form of the gene by means of high throughput genetic sequencing techniques. The application of such techniques to the detection of deletion mutations can be problematic because, among other reasons, the lack of sequences observed in sequencing (as opposed to detecting a simple point mutation or SNP). Such embodiments employ (1) a pair of amplification primers specific for the gene of interest, where in the amplification primers will amplify the gene of interest (or a portion thereof) and will not significantly amplify the mutant allele, (2) a standard sequence corresponding to the wild type allele of the gene of interest(*i.e.*, a target sequence), but differing by at least one detectable nucleotide base, (3) a pair of amplification primers specific for a second target sequence that serves as a reference sequence, and (4) a standard sequence corresponding to the reference sequence.

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[0483] In one embodiment of the invention is provided a method for measuring the number of copies of the gene of interest, where in the gene of interest has one meaning allele that comprises a deletion. The method can employ amplification reagent specific for the gene of interest, *e.g.*, PCR primers, that are specific for the gene of interest by amplifying at least a portion of the gene of interest, or the entire gene of interest, or a region adjacent to the gene of interest, while not amplifying the deletion comprising allele of the gene of interest. Additionally the subject method employs a standard sequence corresponding to the gene of interest, wherein the standard sequence differs by at least one nucleotide base from the gene of interest (so that the sequence of the standard sequence can be readily distinguished from the naturally occurring gene of interest). Typically, the standard sequence will contain the same primer binding sites as the gene of interest so as to minimize any amplification discrimination between the gene of interest and the standard sequence corresponding to the gene of interest. The reaction will also comprises amplification reagents specific for a reference sequence. The reference sequence is a sequence of known (or at least assumed to be known) copy number in the genome to be analyzed. The reaction further comprises a standard sequence corresponding to the reference sequence. Typically, the standard sequence corresponding to the reference sequence will contain the same primer binding sites as the reference sequence so as to minimize any amplification discrimination between the reference sequence and the standard sequence corresponding to the reference sequence.

Exemplary PCR Conditions

[0484] If desired, any of the PCR conditions disclosed herein or any standard PCR conditions can be used to test a primer library to determine, *e.g.*, the percent of primer dimers, percent of target amplicons, and percent of target loci that are amplified. If desired, standard methods can be used to optimize the reaction conditions to improve the performance of a primer library. Any of these PCR conditions may also be used in any of the methods of the invention to amplify target loci. It was determined that high ionic strength solutions can surprisingly be used for multiplex PCR. In some embodiments, monovalent cations are used to increase the ionic strength to, *e.g.*, help the primers bind the template.

[0485] In some embodiments, the reaction volume includes ethylenediaminetetraacetic acid (EDTA), magnesium, tetramethyl ammonium chloride (TMAC), or any combination thereof. In

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some embodiments, the concentration of TMAC is between 20 and 80 mM, such as between 25 and 70 mM, 30 and 60 mM, 30 and 40 mM, 40 and 50 mM, 50 and 60 mM, or 60 and 70 mM, inclusive. While not meant to be bound to any particular theory, it is believed thatTMAC binds to DNA, stabilizes duplexes, increases primer specificity, and/or equalizes the melting temperatures of different primers. In some embodiments, TMAC increases the uniformity in the amount of amplified products for the different targets. In some embodiments, the concentration of magnesium (such as magnesium from magnesium chloride) is between 1 and 10 mM, such as between 1 and 8 mM, 1 and 5 mM, 1 and 3 mM, 3 and 5 mM, 3 and 6 mM, or 5 and 8 mM, inclusive.

[0486] In some embodiments, the concentration of available magnesium (the concentration of magnesium that is assumed to be available for binding the polymerase and not bound to molecules other than the polymerase), such as the magnesium that is not bound by phosphate groups on dNTPs, primers, or nucleic acid templates, or carboxylic acid groups on magnetic or other beads, if present) is between 0.5 to 10 mM, such as between 1 and 8 mM, 1 and 5 mM, 1 and 3 mM, 3 and 5 mM, 3 and 6 mM, 4 and 6 mM, or 5 and 8 mM, inclusive. The large number of primers used for multiplex PCR of a large number of targets may chelate a lot of the magnesium (2 phosphates in the primers chelate 1 magnesium). For example, if enough primers are used such that the concentration of phosphate from the primers is ~9 mM, then the primers may reduce the effective magnesium concentration by ~4.5 mM. In some embodiments, EDTA is used to decrease the amount of magnesium available as a cofactor for the polymerase since high concentrations of magnesium can result in PCR errors, such as amplification of non-target loci. In some embodiments, the concentration of EDTA reduces the amount of available magnesium to between 1 and 5 mM (such as between 3 and 5 mM).

[0487] In some embodiments, the pH is between 7.5 and 8.5, such as between 7.5 and 8, 8 and 8.3, or 8.3 and 8.5, inclusive. In some embodiments, Tris is used at, for example, a concentration of between 10 and 100 mM, such as between 10 and 25 mM, 25 and 50 mM, 50 and 75 mM, or 25 and 75 mM, inclusive. In some embodiments, any of these concentrations of Tris are used at a pH between 7.5 and 8.5. In some embodiments, a combination of KCl and $(NH_4)_2SO_4$ is used, such as between 50 and 150 mM KCl and between 10 and 90 mM $(NH_4)_2SO_4$, inclusive. In some embodiments, the concentration of KCl is between 0 and 30 mM, between 50 and 100 mM, or between 100 and 150 mM, inclusive. In some embodiments, the concentration of $(NH_4)_2SO_4$ is between 10 and 50 mM,50 and 90 mM, 10 and 20 mM, 20 and 40 mM, 40 mM and 60, or 60

mM and 80 mM $(NH_4)_2SO_4$, inclusive. In some embodiments, the ammonium $[NH_4^+]$ concentration is between 0 and 160 mM, such as between 0 to 50, 50 to 100, or 100 to 160 mM, inclusive. In some embodiments, the sum of the potassium and ammonium concentration ($[K^+]$ + $[NH_4^+]$) is between 0 and 160 mM, such as between 0 to 25, 25 to 50, 50 to 150, 50 to 75, 75 to 100, 100 to 125, or 125 to 160 mM, inclusive. An exemplary buffer with $[K^+]$ + $[NH_4^+]$ = 120 mM is 20 mM KCl and 50 mM $(NH_4)_2SO_4$. In some embodiments, the buffer includes 25 to 75 mM Tris, pH 7.2 to 8, 0 to 50 mM KCL, 10 to 80 mM ammonium sulfate, and 3 to 6 mM magnesium, inclusive. In some embodiments, the buffer includes 25 to 75 mM Tris pH 7 to 8.5, 3 to 6 mM $MgCl_2$, 10 to 50 mM KCl, and 20 to 80 mM $(NH_4)_2SO_4$, inclusive. In some embodiments, 100 to 200 Units/mL of polymerase are used. In some embodiments, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 7.5 nM of each primer in the library, 50 mM TMAC, and 7 ul DNA template in a 20 ul final volume at pH 8.1 is used.

[0488] In some embodiments, a crowding agent is used, such as polyethylene glycol (PEG, such as PEG 8,000) or glycerol. In some embodiments, the amount of PEG (such as PEG 8,000) is between 0.1 to 20%, such as between 0.5 to 15%, 1 to 10%, 2 to 8%, or 4 to 8%, inclusive. In some embodiments, the amount of glycerol is between 0.1 to 20%, such as between 0.5 to 15%, 1 to 10%, 2 to 8%, or 4 to 8%, inclusive. In some embodiments, a crowding agent allows either a low polymerase concentration and/or a shorter annealing time to be used. In some embodiments, a crowding agent improves the uniformity of the DOR and/or reduces dropouts (undetected alleles). For example, at 8% PEG, and 50 U/mL polymerase, the uniformity was as good as 150 U/mL polymerase and no PEG. If the error rate increases when PEG is included, a higher magnesium chloride concentration (such greater than or about 4, 5, 6, 7, 8, 9, or 10 $MgCl_2$) can be used to reduce or prevent the increase in error rate. Inclusion of 8% PEG 8,000 allowed successful multiplexing with an annealing time of only 1 minute at an annealing temperature of 63°C.

[0489] In some embodiments, a polymerase with proof-reading activity, a polymerase without (or with negligible) proof-reading activity, or a mixture of a polymerase with proof-reading activity and a polymerase without (or with negligible) proof-reading activity is used. In some embodiments, a hot start polymerase, a non-hot start polymerase, or a mixture of a hot start polymerase and a non-hot start polymerase is used. In some embodiments, a HotStarTaq DNA polymerase is used (see, for example, QIAGEN catalog No. 203203, *see, e.g.*, information available at the World Wide Web at qiagen.com/us/products/catalog/assay-technologies/end-

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point-pcr-and-rt-pcr-reagents/hotstartaq-dna-polymerase/, which is hereby incorporated by reference in its entirety). In some embodiments, AmpliTaq Gold® DNA Polymerase is used; it is a chemically modified form of AmpliTaq® DNA Polymerase requiring thermal activation (see, for example, Applied Biosystems catalog No. N8080241 *see, e.g.*, information available at the World Wide Web at lifetechnologies.com/order/catalog/product/N8080241, which is hereby incorporated by reference in its entirety). In some embodiments, KAPA Taq DNA Polymerase or KAPA Taq HotStart DNA Polymerase is used; they are based on the single-subunit, wild-type *Taq* DNA polymerase of the thermophilic bacterium *Thermus aquaticus*. KAPA Taq and KAPA Taq HotStart DNA Polymerase have 5′-3′ polymerase and 5′-3′ exonuclease activities, but no 3′ to 5′ exonuclease (proofreading) activity (see, for example, KAPA BIOSYSTEMS catalog No. BK1000 *see, e.g.*, information available at the World Wide Web at kapabiosystems.com/product-applications/products/pcr-2/kapa-taq-pcr-kits/, which is hereby incorporated by reference in its entirety). In some embodiments, *Pfu* DNA polymerase is used; it is a highly thermostable DNA polymerase from the hyperthermophilic archaeum *Pyrococcus furiosus*. The enzyme catalyzes the template-dependent polymerization of nucleotides into duplex DNA in the 5'→3' direction. *Pfu* DNA Polymerase also exhibits 3'→5' exonuclease (proofreading) activity that enables the polymerase to correct nucleotide incorporation errors. It has no 5'→3' exonuclease activity (see, for example, Thermo Scientific catalog No. EP0501 *see, e.g.*, information available at the World Wide Web at thermoscientificbio.com/pcr-enzymes-master-mixes-and-reagents/pfu-dna-polymerase/, which is hereby incorporated by reference in its entirety). In some embodiments Klentaq1 is used; it is a Klenow-fragment analog of Taq DNA polymerase, it has no exonuclease or endonuclease activity (see, for example, DNA POLYMERASE TECHNOLOGY, Inc, St. Louis, Missouri, catalog No. 100 *see, e.g.*, information available at the World Wide Web at klentaq.com/products/klentaq, which is hereby incorporated by reference in its entirety). In some embodiments, the polymerase is a PUSHION DNA polymerase, such as PHUSION High Fidelity DNA polymerase (M0530S, New England BioLabs, Inc.) or PHUSION Hot Start Flex DNA polymerase (M0535S, New England BioLabs, Inc.; Frey and Suppman *BioChemica*. 2:34-35, 1995; Chester and Marshak *Analytical Biochemistry*. 209:284-290, 1993, which are each hereby incorporated by reference in its entirety). In some embodiments, the polymerase is a Q5® DNA Polymerase, such as Q5® High-Fidelity DNA Polymerase (M0491S, New England BioLabs, Inc.) or Q5® Hot Start High-Fidelity DNA Polymerase (M0493S, New England BioLabs, Inc.). In some embodiments, the

polymerase is a T4 DNA polymerase (M0203S, New England BioLabs, Inc.; Tabor and Struh. (1989). "DNA-Dependent DNA Polymerases," In Ausebel et al. (Ed.), *Current Protocols in Molecular Biology*. 3.5.10-3.5.12. New York: John Wiley & Sons, Inc., 1989; Sambrook et al. *Molecular Cloning: A Laboratory Manual*. (2nd ed.), 5.44-5.47. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989, which are each hereby incorporated by reference in its entirety).

[0490] In some embodiment, between 5 and 600 Units/mL (Units per 1 mL of reaction volume) of polymerase is used, such as between 5 to 100, 100 to 200, 200 to 300, 300 to 400, 400 to 500, or 500 to 600 Units/mL, inclusive. One unit is commonly defined as the amount of enzyme that will incorporate 15 nmol of dNTP into acid-insoluble material in 30 minutes at 75°C. Exemplary assay conditions for measuring unit activity include 1X THERMOPOL Reaction Buffer, 200 µM dNTPs including [^{3}H]-dTTP and 200 µg/ml activated Calf Thymus DNA (see, *e.g.*, information available at the world wide web at neb.com/products/m0267-taq-dna-polymerase-with-thermopol-buffer, which is hereby incorporated by reference in its entirety). 1X THERMOPOL® Reaction Buffer contains 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% TRITON® X-100, pH 8.8.

[0491] In some embodiments, hot-start PCR is used to reduce or prevent polymerization prior to PCR thermocycling. Exemplary hot-start PCR methods include initial inhibition of the DNA polymerase, or physical separation of reaction components reaction until the reaction mixture reaches the higher temperatures. In some embodiments, the enzyme is spatially separated from the reaction mixture by wax that melts when the reaction reaches high temperature. In some embodiments, slow release of magnesium is used. DNA polymerase requires magnesium ions for activity, so the magnesium is chemically separated from the reaction by binding to a chemical compound, and is released into the solution only at high temperature. In some embodiments, non-covalent binding of an inhibitor is used. In this method a peptide, antibody, or aptamer are non-covalently bound to the enzyme at low temperature and inhibit its activity. After incubation at elevated temperature, the inhibitor is released and the reaction starts. In some embodiments, a cold-sensitive Taq polymerase is used, such as a modified DNA polymerase with almost no activity at low temperature. In some embodiments, chemical modification is used. In this method, a molecule is covalently bound to the side chain of an amino acid in the active site of the DNA polymerase. The molecule is released from the enzyme by incubation of the reaction mixture at elevated temperature. Once the molecule is released, the enzyme is activated.

[0492] In some embodiments, the amount to template nucleic acids (such as an RNA or DNA sample) is between 20 and 5,000 ng, such as between 20 to 200, 200 to 400, 400 to 600, 600 to 1,000; 1,000 to 1,500; or 2,000 to 3,000 ng, inclusive.

[0493] In some embodiments QIAGEN Multiplex PCR Kit is used (QIAGEN catalog No. 206143; *see, e.g.*, information available at the World Wide Web at qiagen.com/products/catalog/assay-technologies/end-point-pcr-and-rt-pcr-reagents/qiagen-multiplex-pcr-kit, which is hereby incorporated by reference in its entirety). For 100 x 50 µl multiplex PCR reactions, the kit includes 2x QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$, 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), and RNase-Free Water (2 x 1.7 ml). The QIAGEN Multiplex PCR Master Mix (MM) contains a combination of KCl and $(NH_4)_2SO_4$ as well as the PCR additive, Factor MP, which increases the local concentration of primers at the template. Factor MP stabilizes specifically bound primers, allowing efficient primer extension by HotStarTaq DNA Polymerase. HotStarTaq DNA Polymerase is a modified form of *Taq* DNA polymerase and has no polymerase activity at ambient temperatures. In some embodiments, HotStarTaq DNA Polymerase is activated by a 15-minute incubation at 95°C which can be incorporated into any existing thermal-cycler program.

[0494] In some embodiments, 1x QIAGEN MM final concentration (the recommended concentration), 7.5 nM of each primer in the library, 50 mM TMAC, and 7 ul DNA template in a 20 ul final volume is used. In some embodiments, the PCR thermocycling conditions include 95°C for 10 minutes (hot start); 20 cycles of 96°C for 30 seconds; 65°C for 15 minutes; and 72°C for 30 seconds; followed by 72°C for 2 minutes (final extension); and then a 4°C hold.

[0495] In some embodiments, 2x QIAGEN MM final concentration (twice the recommended concentration), 2 nM of each primer in the library, 70 mM TMAC, and 7 ul DNA template in a 20 ul total volume is used. In some embodiments, up to 4 mM EDTA is also included. In some embodiments, the PCR thermocycling conditions include 95°C for 10 minutes (hot start); 25 cycles of 96°C for 30 seconds; 65°C for 20 minutes; and 72°C for 30 seconds); followed by 72°C for 2 minutes (final extension); and then a 4°C hold.

[0496] Another exemplary set of PCR thermocyling conditions includes 95°C for 10 minutes, 15 cycles of 95°C for 30 seconds, 65°C for 1 minute, 60°C for 5 minutes, 65°C for 5 minutes and 72°C for 30 seconds; and then 72°C for 2 minutes. In some embodiments, this set of PCR thermocyling conditions is used with the following reaction conditions: 100 mM KCl, 50 mM

$(NH_4)_2SO_4$, 3 mM $MgCl_2$, 7.5 nM of each primer in the library, 50 mM TMAC, and 7 ul DNA template in a 20 ul final volume at pH 8.1.

[0497] Another exemplary set of conditions includes a semi-nested PCR approach. The first PCR reaction uses 20 ul a reaction volume with 2x QIAGEN MM final concentration, 1.875 nM of each primer in the library (outer forward and reverse primers), and DNA template. Thermocycling parameters include 95°C for 10 minutes; 25 cycles of 96°C for 30 seconds, 65°C for 1 minute, 58°C for 6 minutes, 60°C for 8 minutes, 65°C for 4 minutes, and 72°C for 30 seconds; and then 72°C for 2 minutes, and then a 4°C hold. Next, 2 ul of the resulting product, diluted 1:200, is as input in a second PCR reaction. This reaction uses a 10 ul reaction volume with 1x QIAGEN MM final concentration, 20 nM of each inner forward primer, and 1 uM of reverse primer tag. Thermocycling parameters include 95°C for 10 minutes; 15 cycles of 9°5C for 30 seconds, 65°C for 1 minute, 60°C for 5 minutes, 65°C for 5 minutes, and 72°C for 30 seconds; and then 72°C for 2 minutes, and then a 4°C hold.

[0498] Any of the methods disclosed herein or any standard methods can be used to test a primer library to determine, *e.g.*, the percent of primer dimers, percent of target amplicons, and percent of target loci that are amplified. In some embodiments, the PCR products are sequenced as described in Example 15 or using standard sequencing methods. In some embodiments, the percentage of primer dimers can be determined by measuring the number of sequencing reads from primer dimers, the percentage of amplified products that are target amplicons can be determined by measuring the number of sequencing reads that map to target loci; the percent of target loci that are amplified can be determined by measuring the number of target loci for which there are sequencing reads that map to the target loci; the number of copies of a particular amplified target loci can be determined based on the number of sequencing reads that map to that target loci (such as by comparing the number of sequencing reads compared to the sequences reads from a standard of known concentration or amount).

[0499] FIG. 45 contains data (such as percent mapped reads and error rate) from multiplex PCR with various buffers. In this figure, "1xMM" denotes 1x QIAGEN Master Mix (the recommended concentration) discussed above, and "2xMM" denotes 2x QIAGEN Master Mix (twice the recommended concentration). FIG. 45 also lists the components of buffer F-A (also called F-A Gold), F-B (also called F-B Gold), F-D, and F-J (also called F-B Qiagen or F-B Qia) as well as the amount and type of polymerase used to generate the data. FIG. 46 is a graph illustrating the uniformity in DOR for multiplex PCR with buffers from FIG. 45. FIG. 47 is a

graph illustrating the normalized depth of read (DOR) for multiplex PCR with buffers from FIG. 45 with the DOR normalized to that of buffer 2xMM.

Limit of Detection

[0500] As demonstrated by experiments provided in the Examples section, methods provided herein are capable of detecting an average allelic imbalance in a sample with a limit of detection or sensitivity of 0.45% AAI, which is the limit of detection for aneuploidy of an illustrative method of the present invention. Similarly, in certain embodiments, methods provided herein are capable of detecting an average allelic imbalance in a sample of 0.45, 0.5, 0.6, 0.8, 0.8, 0.9, or 1.0%. That is, the test method is capable of detecting chromosomal aneuploidy in a sample down to an AAI of 0.45, 0.5, 0.6, 0.8, 0.8, 0.9, or 1.0%. As demonstrated by experiments provided in the Examples section, methods provided herein are capable of detecting the presence of an SNV in a sample for at least some SNVs, with a limit of detection or sensitivity of 0.2%, which is the limit of detection for at least some SNVs in one illustrative embodiment. Similarly, in certain embodiments, the method is capable of detecting an SNV with a frequency or SNV AAI of 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 0.8, 0.9, or 1.0%. That is, the test method is capable of detecting an SNV in a sample down to a limit of detection of 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 0.8, 0.9, or 1.0% of the total allele counts at the chromosomal locus of the SNV.

[0501] In some embodiments, a limit of detection of a mutation (such as an SNV or CNV) of a method of the invention is less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005%. In some embodiments, a limit of detection of a mutation (such as an SNV or CNV) of a method of the invention is between 15 to 0.005%, such as between 10 to 0.005%, 10 to 0.01%, 10 to 0.1%, 5 to 0.005%, 5 to 0.01%, 5 to 0.1%, 1 to 0.005%, 1 to 0.01%, 1 to 0.1%, 0.5 to 0.005%, 0.5 to 0.01%, 0.5 to 0.1%, or 0.1 to 0.01, inclusive.

[0502] In some embodiments, a limit of detection is such that a mutation (such as an SNV or CNV) that is present in less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules with that locus in a sample (such as a sample of cfDNA or cfRNA) is detected (or is capable of being detected). For example, the mutation can be detected even if less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules that have that locus have that mutation in the locus (instead of, for example, a wild-type or non-mutated version of the locus or a different mutation at that locus). In some embodiments, a limit of detection is such that a mutation (such as an SNV or CNV) that is present in less than or equal

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to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules in a sample (such as a sample of cfDNA or cfRNA) is detected (or is capable of being detected). In some embodiments in which the CNV is a deletion, the deletion can be detected even if it is only present in less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules that have a region of interest that may or may not contain the deletion in a sample. In some embodiments in which the CNV is a deletion, the deletion can be detected even if it is only present in less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules in a sample. In some embodiments in which the CNV is a duplication, the duplication can be detected even if the extra duplicated DNA or RNA that is present is less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules that have a region of interest that may or may not be duplicated in a sample in a sample. In some embodiments in which the CNV is a duplication, the duplication can be detected even if the extra duplicated DNA or RNA that is present is less than or equal to 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, or 0.005% of the DNA or RNA molecules in a sample. Experiment 23 provides exemplary methods for calculating the limit of detection. In some embodiments, the "LOD-zs5.0-mr5" method of Example 23 is used.

Exemplary Nucleic Acid Samples

[0503] In some embodiments, the genetic sample may be prepared and/or purified. There are a number of standard procedures known in the art to accomplish such an end. In some embodiments, the sample may be centrifuged to separate various layers. In some embodiments, the DNA may be isolated using filtration. In some embodiments, the preparation of the DNA may involve amplification, separation, purification by chromatography, liquid separation, isolation, preferential enrichment, preferential amplification, targeted amplification, or any of a number of other techniques either known in the art or described herein.

[0504] In some embodiments, a method disclosed herein could be used in situations where there is a very small amount of DNA present, such as in *in vitro* fertilization, or in forensic situations, where one or a few cells are available (typically less than ten cells, less than twenty cells or less than 40 cells.) In these embodiments, a method disclosed herein serves to make ploidy calls from a small amount of DNA that is not contaminated by other DNA, but where the ploidy calling very difficult the small amount of DNA. In some embodiments, a method disclosed

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herein could be used in situations where the target DNA is contaminated with DNA of another individual, for example in maternal blood in the context of prenatal diagnosis, paternity testing, or products of conception testing. Some other situations where these methods would be particularly advantageous would be in the case of cancer testing where only one or a small number of cells were present among a larger amount of normal cells. The genetic measurements used as part of these methods could be made on any sample comprising DNA or RNA, for example but not limited to: blood, plasma, body fluids, urine, hair, tears, saliva, tissue, skin, fingernails, blastomeres, embryos, fetal cells, amniotic fluid, chorionic villus samples, feces, bile, lymph, cervical mucus, semen, or other cells or materials comprising nucleic acids. In an embodiment, a method disclosed herein could be run with nucleic acid detection methods such as sequencing, microarrays, qPCR, digital PCR, or other methods used to measure nucleic acids. If for some reason it were found to be desirable, the ratios of the allele count probabilities at a locus could be calculated, and the allele ratios could be used to determine ploidy state in combination with some of the methods described herein, provided the methods are compatible. In some embodiments, a method disclosed herein involves calculating, on a computer, allele ratios at the plurality of polymorphic loci from the DNA measurements made on the processed samples. In some embodiments, a method disclosed herein involves calculating, on a computer, allele ratios at the plurality of polymorphic loci from the DNA measurements made on the processed samples along with any combination of other improvements described in this disclosure. Exemplary methods for isolating fetal cells, such as a single fetal cell are disclosed in USSN 61/978648, filed April 11, 2014 and USSN 61/984546, filed April 25, 2014. Fetal cells or fetal nucleic acids can be isolated from a pregnant mother using invasive (such as CVS or amniocentesis) or noninvasive methods (such as from a maternal blood sample).

[0505] In some embodiments, this method may be used to genotype a single cell, a small number of cells, two to five cells, six to ten cells, ten to twenty cells, twenty to fifty cells, fifty to one hundred cells, one hundred to one thousand cells, or a small amount of extracellular DNA, for example from one to ten picograms, from ten to one hundred pictograms, from one hundred pictograms to one nanogram, from one to ten nanograms, from ten to one hundred nanograms, from 30 to 500 nanograms, or from one hundred nanograms to one microgram. In some embodiments, nucleic acids (such as DNA and/or RNA) from less than 100, 75, 50, 40, 30, 20, 10, 8, 6, 4, 2, or 1 cell is amplified with any of the methods of the invention. In some embodiments, the nucleic acid sample includes less than 80, 60, 40, 20, or 10% of the nucleic

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acids (such as DNA and/or RNA) from a single cell. In some embodiments, in which a small number of cells (such as one cell) or a small amount of nucleic acids is used, nested PCR such as hemi-nested or semi-nested PCR is used and/or the number of PCR cycles is increased compare to that used for samples with a larger amount of cells or nucleic acids. In some embodiments, a large amount of cells or nucleic acids are used (such as in cases in which a larger amount is desired to improve performance of any of the methods of the invention. In some embodiments, a sample with at least 2, 5, 10, 15, 20, 30, 50, 100, or more cells (or DNA or RNA from such cells) is used in any of the methods of the invention. In some embodiments, at least 0.5, 1, 10, 25, 50, 100, 500, 1,000; or 5,000 ng of DNA or RNA is used.

[0506] In some embodiments, the cells in the sample are lysed prior to PCR. In some embodiments, the Arcturus PicoPure DNA extraction kit from Applied Biosystems is used. (Applied Biosystems cat. No. KIT0103, *see, e.g.*, information available at the World Wide Web at lifetechnologies.com/order/catalog/product/KIT0103?ICID=search-product, which is hereby incorporated by reference in its entirety). This kit contains Arcturus reconstitution buffer and Protease K. In some embodiments, the following cell lysis thermocycling protocol is used: 56°C for 1 hour, 95°C for 10 minutes, 25°C for 15 minutes, and then a 4°C hold.

[0507] In some embodiments, the nucleic acids are processed using the consecutive steps of end-repairing, dA-tailing, and adaptor ligating the nucleic acids. The consecutive steps exclude purifying the end-repaired products prior to the dA-tailing step and exclude purifying the dA-tailing products prior to the adaptor ligating step. The resulting products are amplified in any of the multiplex PCR methods of the invention. In some embodiments, the amplified products are then sequenced.

Exemplary Nucleic Acid Studies

[0508] The multiplex PCR methods of the invention can be used to increase the number of target loci that can be evaluated to measure the amount of one or more specific nucleic acid molecules of interest or of one or more types of nucleic acids. In some embodiments, there is a change in the total amount or concentration of one or more types of DNA (such as cfDNA cf mDNA, cf nDNA, cellular DNA, or mitochondrial DNA) or RNA (cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA). In some embodiments, there is a change in the amount or concentration

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of one or more specific DNA (such as cfDNA cf mDNA, cf nDNA, cellular DNA, or mitochondrial DNA) or RNA (cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA) molecules. In some embodiments, one allele is expressed more than another allele of a locus of interest. Exemplary miRNAs are short 20–22 nucleotide RNA molecules that regulate the expression of a gene. In some embodiments, there is a change in the transcriptome, such as a change in the identity or amount of one or more RNA molecules.

[0509] In some embodiments, an increase in the total amount or concentration of cfDNA or cfRNA is associated with a disease such as cancer, or an increased risk for a disease such as cancer. In some embodiments, the total concentration of a type of DNA (such as cfDNA cf mDNA, cf nDNA, cellular DNA, or mitochondrial DNA) or RNA (cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA) increases by at least 2, 3, 4, 5, 6, 7, 8, 9, 10-fold, or more compared to the total concentration of that type of DNA or RNA in healthy (such as non-cancerous) subjects. In some embodiments, a total concentration of cfDNA between 75 to 100 ng/mL, 100 to 150 ng/mL, 150 to 200 ng/mL, 200 to 300 ng/mL, 300 to 400 ng/mgL, 400 to 600 ng/mL, 600 to 800 ng/mL, 800 to 1,000 ng/mL, inclusive, or a total concentration of cfDNA of more than 100 ng, mL, such as more than 200, 300, 400, 500, 600, 700, 800, 900, or 1,000 ng/mL is indicative of cancer, an increased risk for cancer, an increased risk of a tumor being malignant rather than benign, a decreased probably of the cancer going into remission, or a worse prognosis for the cancer. In some embodiments, the amount of a type of DNA (such as cfDNA cf mDNA, cf nDNA, cellular DNA, or mitochondrial DNA) or RNA (cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA) having one or more polymorphisms/mutations (such as deletions or duplications) associated with a disease such as cancer or an increased risk for a disease such as cancer is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, or 25% of the total amount of that type of DNA or RNA. In some embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, or 25% of the total amount of a type of DNA (such as cfDNA cf mDNA, cf nDNA, cellular DNA, or mitochondrial DNA) or RNA (cfRNA, cellular RNA, cytoplasmic RNA, coding cytoplasmic RNA, non-coding cytoplasmic RNA, mRNA, miRNA, mitochondrial RNA, rRNA, or tRNA) has a particular polymorphism or mutation (such as a deletion or duplication) associated with a disease such as cancer or an increased risk for a disease such as cancer.

Exemplary RNA Expression Studies

[0510] The multiplex PCR methods of the invention can be used to increase the number of target loci that can be evaluated during gene expression profiling experiments. For example, the expression levels of thousands of genes can be simultaneously monitored to determine whether a person has a sequence (such as a polymorphism or other mutation) associated with a disease (such as cancer) or an increased risk of a disease. These methods can be used to identify sequences (such as polymorphisms or other mutations) associated with an increased or decreased risk for a disease such as cancer by comparing gene expression (such as the expression of particular mRNA alleles) in samples from patients with and without the disease. Additionally, the effect of particular treatments, diseases, or developmental stages on gene expression can be determined. Similarly, these methods can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing gene expression in infected and uninfected cells or tissues. In these methods the number of sequencing reads can be adjusted based on the frequency of the polymorphisms that are being analyzed such that sufficient reads are performed for the polymorphisms to be detected if they are present. In some embodiments, the polymorphisms or mutation is present at a higher frequency in subjects with a disease or disorder (such as cancer) than subjects without the disease or disorder (such as cancer). In some embodiments, the polymorphisms or mutation is indicative of cancer, such as a causative mutation.

[0511] In some embodiments, a sample containing RNA (such as mRNA) is amplified using a reverse transcriptase (RT) and the resulting DNA (such as cDNA) is then amplified using a DNA polymerase (PCR). The RT and PCR steps may be carried out sequentially in the same reaction volume or separately. Any of the primer libraries of the invention can be used in this reverse transcription polymerase chain reaction (RT-PCR) method. In various embodiments, the reverse transcription is performed using oligo-dT, random primers, a mixture of oligo-dT and random primers, or primers specific to the target loci. To avoid amplification of contaminating genomic DNA, primers for RT-PCR can be designed so that part of one primer hybridizes to the 3' end of one exon and the other part of the primer hybridizes to the 5' end of the adjacent exon. Such primers anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA. To detect amplification of contaminating DNA, RT-PCR primer pairs may be designed to flank a region that contains at least one intron. Products amplified from cDNA (no introns) are smaller than

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those amplified from genomic DNA (containing introns). Size difference in products is used to detect the presence of contaminating DNA. In some embodiments when only the mRNA sequence is known, primer annealing sites are chosen that are at least 300–400 base pairs apart since it is likely that fragments of this size from eukaryotic DNA contain splice junctions. Alternatively, the sample can be treated with DNase to degrade contaminating DNA.

Exemplary Methods for Paternity Testing

[0512] The multiplex PCR methods of the invention can be used to improve the accuracy of paternity testing since so many target loci can be analyzed at once (see, *e.g*, U.S. Publication No. 2012/0122701, filed December 22, 2011, is which is hereby incorporated by reference in its entirety). For example, the multiplex PCR method can allow thousands of polymorphic loci (such as SNPs) to be analyzed for use in the PARENTAL SUPPORT algorithm described herein to determine whether an alleged father in is the biological father of a fetus. In some embodiments the method involves (i) simultaneously amplifying a plurality of polymorphic loci that includes at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different polymorphic loci on genetic material from the alleged father to produce a first set of amplified products; (ii) simultaneously amplifying the corresponding plurality of polymorphic loci on a mixed sample of DNA originating from a blood sample from the pregnant mother to produce a second set of amplified products; wherein the mixed sample of DNA comprises fetal DNA and maternal DNA; (iii) determining on a computer the probability that the alleged father is the biological father of the fetus using genotypic measurements based on the first and second sets of amplified products; and (iv) establishing whether the alleged father is the biological father of the fetus using the determined probability that the alleged father is the biological father of the fetus. In various embodiments, the method further includes simultaneously amplifying the corresponding plurality of polymorphic loci on genetic material from the mother to produce a third set of amplified products; wherein the probability that the alleged father is the biological father of the fetus is determined using genotypic measurements based on the first, second, and third sets of amplified products.

Exemplary Methods for Embryo Characterization and Selection

[0513] The multiplex PCR methods of the invention can be used to improve the selection of embryos for *in vitro* fertilization by allowing thousands of target loci to be analyzed at once (*see, e.g*, U.S. Pub. No. 2011/0092763, filed May 27, 2008, filed December 22, 2011, is which is hereby incorporated by reference in its entirety). For example, the multiplex PCR method can allow thousands of polymorphic loci (such as SNPs) to be analyzed for use in the PARENTAL SUPPORT algorithm described herein to select an embryo out of a set of embryos for *in vitro* fertilization

[0514] In some embodiments, the invention provides methods of estimating relative likelihoods that each embryo from a set of embryos will develop as desired. In some embodiments, the method involves contacting a sample from each embryo with a library of primers that simultaneously hybridize to at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 27,000; 28,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture for each embryo, wherein the samples are each derived from one or more cells from an embryo. In some embodiments, each reaction mixture is subjected to primer extension reaction conditions to produce amplified products. In some embodiments, the method includes determining on a computer one or more characteristics of at least one cell from each embryo based on the amplified products; and estimating on a computer the relative likelihoods that each embryo will develop as desired, based on the one or more characteristics of the at least one cell for each embryo. In some embodiments, the method includes using an informatics based method to determine the at least one characteristic, such as the PARENTAL SUPPORT algorithm described herein. In some embodiments, the characteristic includes a ploidy state. In some embodiments, the characteristic is selected from the group consisting of aneuploid, euploid, mosaic, nullsomy, monosomy, uniparental disomy, trisomy, tetrasomy, a type of aneuploidy, unmatched copy error trisomy, matched copy error trisomy, maternal origin of aneuploidy, paternal origin of aneuploidy, a presence or absence of a disease-linked gene, a chromosomal identity of any aneuploid chromosome, an abnormal genetic condition, a deletion or duplication, a likelihood of a characteristic, and combinations thereof. The characteristic may be associated with a chromosome taken from the group consisting of chromosome one, chromosome two, chromosome three, chromosome four, chromosome five, chromosome six, chromosome seven, chromosome eight, chromosome nine, chromosome ten, chromosome eleven, chromosome twelve, chromosome thirteen, chromosome fourteen, chromosome fifteen, chromosome sixteen, chromosome seventeen, chromosome

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eighteen, chromosome nineteen, chromosome twenty, chromosome twenty-one, chromosome twenty-two, X chromosome or Y chromosome, and combinations thereof.

Exemplary Prenatal Diagnostic Methods

[0515] The multiplex PCR methods of the present invention can be used to improve prenatal diagnostic methods, such as the determination of the ploidy status of fetal chromosomes. Given that the large number of target loci that can be simultaneously amplified, more accurate determinations can be made.

[0516] In an embodiment, the present disclosure provides *ex vivo* methods for determining the ploidy status of a chromosome in a gestating fetus from genotypic data measured from a mixed sample of DNA (*i.e.*, DNA from the mother of the fetus, and DNA from the fetus) and optionally from genotypic data measured from a sample of genetic material from the mother and possibly also from the father, wherein the determining is done by using a joint distribution model to create a set of expected allele distributions for different possible fetal ploidy states given the parental genotypic data, and comparing the expected allelic distributions to the actual allelic distributions measured in the mixed sample, and choosing the ploidy state whose expected allelic distribution pattern most closely matches the observed allelic distribution pattern. In an embodiment, the mixed sample is derived from maternal blood, or maternal serum or plasma. In an embodiment, the mixed sample of DNA may be preferentially enriched at a target loci (*e.g.*, plurality of polymorphic loci). In an embodiment, the preferential enrichment is done in a way that minimizes the allelic bias. In an embodiment, the present disclosure relates to a composition of DNA that has been preferentially enriched at a plurality of loci such that the allelic bias is low. In an embodiment, the allelic distribution(s) are measured by sequencing the DNA from the mixed sample. In an embodiment, the joint distribution model assumes that the alleles will be distributed in a binomial fashion. In an embodiment, the set of expected joint allele distributions are created for genetically linked loci while considering the extant recombination frequencies from various sources, for example, using data from the International HapMap Consortium.

[0517] In an embodiment, the present disclosure provides methods for non-invasive prenatal diagnosis (NPD), specifically, determining the aneuploidy status of a fetus by observing allele measurements at a plurality of polymorphic loci in genotypic data measured on DNA mixtures, where certain allele measurements are indicative of an aneuploid fetus, while other allele

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measurements are indicative of a euploid fetus. In an embodiment, the genotypic data is measured by sequencing DNA mixtures that were derived from maternal plasma. In an embodiment, the DNA sample may be preferentially enriched in molecules of DNA that correspond to the plurality of loci whose allele distributions are being calculated. In an embodiment a sample of DNA comprising only or almost only genetic material from the mother and possibly also a sample of DNA comprising only or almost only genetic material from the father are measured. In an embodiment, the genetic measurements of one or both parents along with the estimated fetal fraction are used to create a plurality of expected allele distributions corresponding to different possible underlying genetic states of the fetus; the expected allele distributions may be termed hypotheses. In an embodiment, the maternal genetic data is not determined by measuring genetic material that is exclusively or almost exclusively maternal in nature, rather, it is estimated from the genetic measurements made on maternal plasma that comprises a mixture of maternal and fetal DNA. In some embodiments the hypotheses may comprise the ploidy of the fetus at one or more chromosomes, which segments of which chromosomes in the fetus were inherited from which parents, and combinations thereof. In some embodiments, the ploidy state of the fetus is determined by comparing the observed allele measurements to the different hypotheses where at least some of the hypotheses correspond to different ploidy states, and selecting the ploidy state that corresponds to the hypothesis that is most likely to be true given the observed allele measurements. In an embodiment, this method involves using allele measurement data from some or all measured SNPs, regardless of whether the loci are homozygous or heterozygous, and therefore does not involve using alleles at loci that are only heterozygous. This method may not be appropriate for situations where the genetic data pertains to only one polymorphic locus. This method is particularly advantageous when the genetic data comprises data for more than ten polymorphic loci for a target chromosome or more than twenty polymorphic loci. This method is especially advantageous when the genetic data comprises data for more than 50 polymorphic loci for a target chromosome, more than 100 polymorphic loci or more than 200 polymorphic loci for a target chromosome. In some embodiments, the genetic data may comprise data for more than 500 polymorphic loci for a target chromosome, more than 1,000 polymorphic loci, more than 2,000 polymorphic loci, or more than 5,000 polymorphic loci for a target chromosome.

[0518] In an embodiment, a method disclosed herein yields a quantitative measure of the number of independent observations of each allele at a polymorphic locus. This is unlike most methods

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such as microarrays or qualitative PCR which provide information about the ratio of two alleles but do not quantify the number of independent observations of either allele. With methods that provide quantitative information regarding the number of independent observations, only the ratio is utilized in ploidy calculations, while the quantitative information by itself is not useful. To illustrate the importance of retaining information about the number of independent observations consider the sample locus with two alleles, A and B. In a first experiment twenty A alleles and twenty B alleles are observed, in a second experiment 200 A alleles and 200 B alleles are observed. In both experiments the ratio (A/(A+B)) is equal to 0.5, however the second experiment conveys more information than the first about the certainty of the frequency of the A or B allele. Some methods by others involve averaging or summing allele ratios (channel ratios) (*i.e.* x_i/y_i) from individual allele and analyzes this ratio, either comparing it to a reference chromosome or using a rule pertaining to how this ratio is expected to behave in particular situations. No allele weighting is implied in such methods, where it is assumed that one can ensure about the same amount of PCR product for each allele and that all the alleles should behave the same way. Such a method has a number of disadvantages, and more importantly, precludes the use a number of improvements that are described elsewhere in this disclosure.

[0519] In an embodiment, a method disclosed herein explicitly models the allele frequency distributions expected in disomy as well as a plurality of allele frequency distributions that may be expected in cases of trisomy resulting from nondisjunction during meiosis I, nondisjunction during meiosis II, and/or nondisjunction during mitosis early in fetal development. To illustrate why this is important, imagine a case where there were no crossovers: nondisjunction during meiosis I would result a trisomy in which two different homologs were inherited from one parent; in contrast, nondisjunction during meiosis II or during mitosis early in fetal development would result in two copies of the same homolog from one parent. Each scenario would result in different expected allele frequencies at each polymorphic locus and also at all loci considered jointly, due to genetic linkage. Crossovers, which result in the exchange of genetic material between homologs, make the inheritance pattern more complex; in an embodiment, the instant method accommodates for this by using recombination rate information in addition to the physical distance between loci. In an embodiment, to enable improved distinction between meiosis I nondisjunction and meiosis II or mitotic nondisjunction the instant method incorporate into the model an increasing probability of crossover as the distance from the centromere increases. Meiosis II and mitotic nondisjunction can distinguished by the fact that mitotic

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nondisjunction typically results in identical or nearly identical copies of one homolog while the two homologs present following a meiosis II nondisjunction event often differ due to one or more crossovers during gametogenesis.

[0520] In some embodiments, a method disclosed herein involves comparing the observed allele measurements to theoretical hypotheses corresponding to possible fetal genetic aneuploidy, and does not involve a step of quantitating a ratio of alleles at a heterozygous locus. Where the number of loci is lower than about 20, the ploidy determination made using a method comprising quantitating a ratio of alleles at a heterozygous locus and a ploidy determination made using a method comprising comparing the observed allele measurements to theoretical allele distribution hypotheses corresponding to possible fetal genetic states may give a similar result. However, where the number of loci is above 50 these two methods is likely to give significantly different results; where the number of loci is above 400, above, 1,000 or above 2,000 these two methods are very likely to give results that are increasingly significantly different. These differences are due to the fact that a method that comprises quantitating a ratio of alleles at a heterozygous locus without measuring the magnitude of each allele independently and aggregating or averaging the ratios precludes the use of techniques including using a joint distribution model, performing a linkage analysis, using a binomial distribution model, and/or other advanced statistical techniques, whereas using a method comprising comparing the observed allele measurements to theoretical allele distribution hypotheses corresponding to possible fetal genetic states may use these techniques which can substantially increase the accuracy of the determination.

[0521] In an embodiment, a method disclosed herein involves determining whether the distribution of observed allele measurements is indicative of a euploid or an aneuploid fetus using a joint distribution model. The use of a joint distribution model is a different from and a significant improvement over methods that determine heterozygosity rates by treating polymorphic loci independently in that the resultant determinations are of significantly higher accuracy. Without being bound by any particular theory, it is believed that one reason they are of higher accuracy is that the joint distribution model takes into account the linkage between SNPs, and likelihood of crossovers having occurred during the meiosis that gave rise to the gametes that formed the embryo that grew into the fetus. The purpose of using the concept of linkage when creating the expected distribution of allele measurements for one or more hypotheses is that it allows the creation of expected allele measurements distributions that correspond to reality considerably better than when linkage is not used. For example, imagine

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that there are two SNPs, 1 and 2 located nearby one another, and the mother is A at SNP 1 and A at SNP 2 on one homolog, and B at SNP 1 and B at SNP 2 on homolog two. If the father is A for both SNPs on both homologs, and a B is measured for the fetus SNP 1, this indicates that homolog two has been inherited by the fetus, and therefore that there is a much higher likelihood of a B being present on the fetus at SNP 2. A model that takes into account linkage would predict this, while a model that does not take linkage into account would not. Alternately, if a mother was AB at SNP 1 and AB at nearby SNP 2, then two hypotheses corresponding to maternal trisomy at that location could be used – one involving a matching copy error (nondisjunction in meiosis II or mitosis in early fetal development), and one involving an unmatching copy error (nondisjunction in meiosis I). In the case of a matching copy error trisomy, if the fetus inherited an AA from the mother at SNP 1, then the fetus is much more likely to inherit either an AA or BB from the mother at SNP 2, but not AB. In the case of an unmatching copy error, the fetus would inherit an AB from the mother at both SNPs. The allele distribution hypotheses made by a ploidy calling method that takes into account linkage would make these predictions, and therefore correspond to the actual allele measurements to a considerably greater extent than a ploidy calling method that did not take into account linkage. Note that a linkage approach is not possible when using a method that relies on calculating allele ratios and aggregating those allele ratios.

[0522] One reason that it is believed that ploidy determinations that use a method that comprises comparing the observed allele measurements to theoretical hypotheses corresponding to possible fetal genetic states are of higher accuracy is that when sequencing is used to measure the alleles, this method can glean more information from data from alleles where the total number of reads is low than other methods; for example, a method that relies on calculating and aggregating allele ratios would produce disproportionately weighted stochastic noise. For example, imagine a case that involved measuring the alleles using sequencing, and where there was a set of loci where only five sequence reads were detected for each locus. In an embodiment, for each of the alleles, the data may be compared to the hypothesized allele distribution, and weighted according to the number of sequence reads; therefore the data from these measurements would be appropriately weighted and incorporated into the overall determination. This is in contrast to a method that involved quantitating a ratio of alleles at a heterozygous locus, as this method could only calculate ratios of 0%, 20%, 40%, 60%, 80% or 100% as the possible allele ratios; none of these may be close to expected allele ratios. In this latter case, the calculated allele rations would either

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have to be discarded due to insufficient reads or else would have disproportionate weighting and introduce stochastic noise into the determination, thereby decreasing the accuracy of the determination. In an embodiment, the individual allele measurements may be treated as independent measurements, where the relationship between measurements made on alleles at the same locus is no different from the relationship between measurements made on alleles at different loci.

[0523] In an embodiment, a method disclosed herein involves determining whether the distribution of observed allele measurements is indicative of a euploid or an aneuploid fetus without comparing any metrics to observed allele measurements on a reference chromosome that is expected to be disomic (termed the RC method). This is a significant improvement over methods, such as methods using shotgun sequencing which detect aneuploidy by evaluating the proportion of randomly sequenced fragments from a suspect chromosomes relative to one or more presumed disomic reference chromosome. This RC method yields incorrect results if the presumed disomic reference chromosome is not actually disomic. This can occur in cases where aneuploidy is more substantial than trisomy of a single chromosome or where the fetus is triploid and all autosomes are trisomic. In the case of a female triploid (69, XXX) fetus there are in fact no disomic chromosomes at all. The method described herein does not require a reference chromosome and would be able to correctly identify trisomic chromosomes in a female triploid fetus. For each chromosome, hypothesis, child fraction and noise level, a joint distribution model may be fit, without any of: reference chromosome data, an overall child fraction estimate, or a fixed reference hypothesis.

[0524] In an embodiment, a method disclosed herein demonstrates how observing allele distributions at polymorphic loci can be used to determine the ploidy state of a fetus with greater accuracy than methods in the prior art. In an embodiment, the method uses the targeted sequencing to obtain mixed maternal-fetal genotypes and optionally mother and/or father genotypes at a plurality of SNPs to first establish the various expected allele frequency distributions under the different hypotheses, and then observing the quantitative allele information obtained on the maternal-fetal mixture and evaluating which hypothesis fits the data best, where the genetic state corresponding to the hypothesis with the best fit to the data is called as the correct genetic state. In an embodiment, a method disclosed herein also uses the degree of fit to generate a confidence that the called genetic state is the correct genetic state. In an embodiment, a method disclosed herein involves using algorithms that analyze the distribution

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of alleles found for loci that have different parental contexts, and comparing the observed allele distributions to the expected allele distributions for different ploidy states for the different parental contexts (different parental genotypic patterns). This is different from and an improvement over methods that do not use methods that enable the estimation of the number of independent instances of each allele at each locus in a mixed maternal-fetal sample. In an embodiment, a method disclosed herein involves determining whether the distribution of observed allele measurements is indicative of a euploid or an aneuploid fetus using observed allelic distributions measured at loci where the mother is heterozygous. This is different from and an improvement over methods that do not use observed allelic distributions at loci where the mother is heterozygous because, in cases where the DNA is not preferentially enriched or is preferentially enriched for loci that are not known to be highly informative for that particular target individual, it allows the use of about twice as much genetic measurement data from a set of sequence data in the ploidy determination, resulting in a more accurate determination.

[0525] In an embodiment, a method disclosed herein uses a joint distribution model that assumes that the allele frequencies at each locus are multinomial (and thus binomial when SNPs are biallelic) in nature. In some embodiments the joint distribution model uses beta-binomial distributions. When using a measuring technique, such as sequencing, provides a quantitative measure for each allele present at each locus, binomial model can be applied to each locus and the degree underlying allele frequencies and the confidence in that frequency can be ascertained. With methods known in the art that generate ploidy calls from allele ratios, or methods in which quantitative allele information is discarded, the certainty in the observed ratio cannot be ascertained. The instant method is different from and an improvement over methods that calculate allele ratios and aggregate those ratios to make a ploidy call, since any method that involves calculating an allele ratio at a particular locus, and then aggregating those ratios, necessarily assumes that the measured intensities or counts that are indicative of the amount of DNA from any given allele or locus will be distributed in a Gaussian fashion. The method disclosed herein does not involve calculating allele ratios. In some embodiments, a method disclosed herein may involve incorporating the number of observations of each allele at a plurality of loci into a model. In some embodiments, a method disclosed herein may involve calculating the expected distributions themselves, allowing the use of a joint binomial distribution model which may be more accurate than any model that assumes a Gaussian distribution of allele measurements. The likelihood that the binomial distribution model is

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significantly more accurate than the Gaussian distribution increases as the number of loci increases. For example, when fewer than 20 loci are interrogated, the likelihood that the binomial distribution model is significantly better is low. However, when more than 100, or especially more than 400, or especially more than 1,000, or especially more than 2,000 loci are used, the binomial distribution model will have a very high likelihood of being significantly more accurate than the Gaussian distribution model, thereby resulting in a more accurate ploidy determination. The likelihood that the binomial distribution model is significantly more accurate than the Gaussian distribution also increases as the number of observations at each locus increases. For example, when fewer than 10 distinct sequences are observed at each locus are observed, the likelihood that the binomial distribution model is significantly better is low. However, when more than 50 sequence reads, or especially more than 100 sequence reads, or especially more than 200 sequence reads, or especially more than 300 sequence reads are used for each locus, the binomial distribution model will have a very high likelihood of being significantly more accurate than the Gaussian distribution model, thereby resulting in a more accurate ploidy determination.

[0526] In an embodiment, a method disclosed herein uses sequencing to measure the number of instances of each allele at each locus in a DNA sample. Each sequencing read may be mapped to a specific locus and treated as a binary sequence read; alternately, the probability of the identity of the read and/or the mapping may be incorporated as part of the sequence read, resulting in a probabilistic sequence read, that is, the probable whole or fractional number of sequence reads that map to a given loci. Using the binary counts or probability of counts it is possible to use a binomial distribution for each set of measurements, allowing a confidence interval to be calculated around the number of counts. This ability to use the binomial distribution allows for more accurate ploidy estimations and more precise confidence intervals to be calculated. This is different from and an improvement over methods that use intensities to measure the amount of an allele present, for example methods that use microarrays, or methods that make measurements using fluorescence readers to measure the intensity of fluorescently tagged DNA in electrophoretic bands.

[0527] In an embodiment, a method disclosed herein uses aspects of the present set of data to determine parameters for the estimated allele frequency distribution for that set of data. This is an improvement over methods that utilize training set of data or prior sets of data to set parameters for the present expected allele frequency distributions, or possibly expected allele

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ratios. This is because there are different sets of conditions involved in the collection and measurement of every genetic sample, and thus a method that uses data from the instant set of data to determine the parameters for the joint distribution model that is to be used in the ploidy determination for that sample will tend to be more accurate.

[0528] In an embodiment, a method disclosed herein involves determining whether the distribution of observed allele measurements is indicative of a euploid or an aneuploid fetus using a maximum likelihood technique. The use of a maximum likelihood technique is different from and a significant improvement over methods that use single hypothesis rejection technique in that the resultant determinations will be made with significantly higher accuracy. One reason is that single hypothesis rejection techniques set cut off thresholds based on only one measurement distribution rather than two, meaning that the thresholds are usually not optimal. Another reason is that the maximum likelihood technique allows the optimization of the cut off threshold for each individual sample instead of determining a cut off threshold to be used for all samples regardless of the particular characteristics of each individual sample. Another reason is that the use of a maximum likelihood technique allows the calculation of a confidence for each ploidy call. The ability to make a confidence calculation for each call allows a practitioner to know which calls are accurate, and which are more likely to be wrong. In some embodiments, a wide variety of methods may be combined with a maximum likelihood estimation technique to enhance the accuracy of the ploidy calls. In an embodiment, the maximum likelihood technique may be used in combination with the method described in US Patent 7,888,017. In an embodiment, the maximum likelihood technique may be used in combination with the method of using targeted PCR amplification to amplify the DNA in the mixed sample followed by sequencing and analysis using a read counting method such as used by TANDEM DIAGNOSTICS, as presented at the International Congress of Human Genetics 2011, in Montreal in October 2011. In an embodiment, a method disclosed herein involves estimating the fetal fraction of DNA in the mixed sample and using that estimation to calculate both the ploidy call and the confidence of the ploidy call. Note that this is both different and distinct from methods that use estimated fetal fraction as a screen for sufficient fetal fraction, followed by a ploidy call made using a single hypothesis rejection technique that does not take into account the fetal fraction nor does it produce a confidence calculation for the call.

[0529] In an embodiment, a method disclosed herein takes into account the tendency for the data to be noisy and contain errors by attaching a probability to each measurement. The use of

maximum likelihood techniques to choose the correct hypothesis from the set of hypotheses that were made using the measurement data with attached probabilistic estimates makes it more likely that the incorrect measurements will be discounted, and the correct measurements will be used in the calculations that lead to the ploidy call. To be more precise, this method systematically reduces the influence of data that is incorrectly measured on the ploidy determination. This is an improvement over methods where all data is assumed to be equally correct or methods where outlying data is arbitrarily excluded from calculations leading to a ploidy call. Existing methods using channel ratio measurements claim to extend the method to multiple SNPs by averaging individual SNP channel ratios. Not weighting individual SNPs by expected measurement variance based on the SNP quality and observed depth of read reduces the accuracy of the resulting statistic, resulting in a reduction of the accuracy of the ploidy call significantly, especially in borderline cases.

[0530] In an embodiment, a method disclosed herein does not presuppose the knowledge of which SNPs or other polymorphic loci are heterozygous on the fetus. This method allows a ploidy call to be made in cases where paternal genotypic information is not available. This is an improvement over methods where the knowledge of which SNPs are heterozygous must be known ahead of time in order to appropriately select loci to target, or to interpret the genetic measurements made on the mixed fetal/maternal DNA sample.

[0531] The methods described herein are particularly advantageous when used on samples where a small amount of DNA is available, or where the percent of fetal DNA is low. This is due to the correspondingly higher allele dropout rate that occurs when only a small amount of DNA is available and/or the correspondingly higher fetal allele dropout rate when the percent of fetal DNA is low in a mixed sample of fetal and maternal DNA. A high allele dropout rate, meaning that a large percentage of the alleles were not measured for the target individual, results in poorly accurate fetal fractions calculations, and poorly accurate ploidy determinations. Since methods disclosed herein may use a joint distribution model that takes into account the linkage in inheritance patterns between SNPs, significantly more accurate ploidy determinations may be made. The methods described herein allow for an accurate ploidy determination to be made when the percent of molecules of DNA that are fetal in the mixture is less than 40%, less than 30%, less than 20%, less than 10%, less than 8%, and even less than 6%.

[0532] In an embodiment, it is possible to determine the ploidy state of an individual based on measurements when that individual's DNA is mixed with DNA of a related individual. In an

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embodiment, the mixture of DNA is the free floating DNA found in maternal plasma, which may include DNA from the mother, with known karyotype and known genotype, and which may be mixed with DNA of the fetus, with unknown karyotype and unknown genotype. It is possible to use the known genotypic information from one or both parents to predict a plurality of potential genetic states of the DNA in the mixed sample for different ploidy states, different chromosome contributions from each parent to the fetus, and optionally, different fetal DNA fractions in the mixture. Each potential composition may be referred to as a hypothesis. The ploidy state of the fetus can then be determined by looking at the actual measurements, and determining which potential compositions are most likely given the observed data.

[0533] Further discussion of the points above may be found elsewhere in this document.

Non-Invasive Prenatal Diagnosis (NPD)

[0534] The process of non-invasive prenatal diagnosis involves a number of steps. Some of the steps may include: (1) obtaining the genetic material from the fetus; (2) enriching the genetic material of the fetus that may be in a mixed sample, *ex vivo*; (3) amplifying the genetic material, *ex vivo*; (4) preferentially enriching specific loci in the genetic material, *ex vivo*; (5) measuring the genetic material, *ex vivo*; and (6) analyzing the genotypic data, on a computer, and *ex vivo*. Methods to reduce to practice these six and other relevant steps are described herein. At least some of the method steps are not directly applied on the body. In an embodiment, the present disclosure relates to methods of treatment and diagnosis applied to tissue and other biological materials isolated and separated from the body. At least some of the method steps are executed on a computer.

[0535] Some embodiments of the present disclosure allow a clinician to determine the genetic state of a fetus that is gestating in a mother in a non-invasive manner such that the health of the baby is not put at risk by the collection of the genetic material of the fetus, and that the mother is not required to undergo an invasive procedure. Moreover, in certain aspects, the present disclosure allows the fetal genetic state to be determined with high accuracy, significantly greater accuracy than, for example, the non-invasive maternal serum analyte based screens, such as the triple test, that are in wide use in prenatal care.

[0536] The high accuracy of the methods disclosed herein is a result of an informatics approach to analysis of the genotype data, as described herein. Modern technological advances have

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resulted in the ability to measure large amounts of genetic information from a genetic sample using such methods as high throughput sequencing and genotyping arrays. The methods disclosed herein allow a clinician to take greater advantage of the large amounts of data available, and make a more accurate diagnosis of the fetal genetic state. The details of a number of embodiments are given below. Different embodiments may involve different combinations of the aforementioned steps. Various combinations of the different embodiments of the different steps may be used interchangeably.

[0537] In an embodiment, a blood sample is taken from a pregnant mother, and the free floating DNA in the plasma of the mother's blood, which contains a mixture of both DNA of maternal origin, and DNA of fetal origin, is isolated and used to determine the ploidy status of the fetus. In an embodiment, a method disclosed herein involves preferential enrichment of those DNA sequences in a mixture of DNA that correspond to polymorphic alleles in a way that the allele ratios and/or allele distributions remain mostly consistent upon enrichment. In an embodiment, a method disclosed herein involves the highly efficient targeted PCR based amplification such that a very high percentage of the resulting molecules correspond to targeted loci. In an embodiment, a method disclosed herein involves sequencing a mixture of DNA that contains both DNA of maternal origin, and DNA of fetal origin. In an embodiment, a method disclosed herein involves using measured allele distributions to determine the ploidy state of a fetus that is gestating in a mother. In an embodiment, a method disclosed herein involves reporting the determined ploidy state to a clinician. In an embodiment, a method disclosed herein involves taking a clinical action, for example, performing follow up invasive testing such as chorionic villus sampling or amniocentesis, preparing for the birth of a trisomic individual or an elective termination of a trisomic fetus.

[0538] This application makes reference to U.S. Utility Application Serial No. 11/603,406, filed November 28, 2006 (US Publication No.: 20070184467); U.S. Utility Application Serial No. 12/076,348, filed March 17, 2008 (US Publication No.: 20080243398); PCT Application Serial No. PCT/US09/52730, filed August 4, 2009 (PCT Publication No.: WO/2010/017214); PCT Application Serial No. PCT/US10/050824, filed September 30, 2010 (PCT Publication No.: WO/2011/041485), U.S. Utility Application Serial No. 13/110,685, filed May 18, 2011, and PCT Application Serial No. PCT/12/58578, filed October 3, 2012, which are each herein incorporated by reference in its entirety. Some of the vocabulary used in this filing may have its

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antecedents in these references. Some of the concepts described herein may be better understood in light of the concepts found in these references.

Screening Maternal Blood Comprising Free Floating Fetal DNA

[0539] The methods described herein may be used to help determine the genotype of a child, fetus, or other target individual where the genetic material of the target is found in the presence of a quantity of other genetic material. In some embodiments the genotype may refer to the ploidy state of one or a plurality of chromosomes, it may refer to one or a plurality of disease linked alleles, or some combination thereof. In this disclosure, the discussion focuses on determining the genetic state of a fetus where the fetal DNA is found in maternal blood, but this example is not meant to limit to possible contexts that this method may be applied to. In addition, the method may be applicable in cases where the amount of target DNA is in any proportion with the non-target DNA; for example, the target DNA could make up anywhere between 0.000001 and 99.999999% of the DNA present. In addition, the non-target DNA does not necessarily need to be from one individual, or even from a related individual, as long as genetic data from some or all of the relevant non-target individual(s) is known. In an embodiment, a method disclosed herein can be used to determine genotypic data of a fetus from maternal blood that contains fetal DNA. It may also be used in a case where there are multiple fetuses in the uterus of a pregnant woman, or where other contaminating DNA may be present in the sample, for example from other already born siblings.

[0540] This technique may make use of the phenomenon of fetal blood cells gaining access to maternal circulation through the placental villi. Ordinarily, only a very small number of fetal cells enter the maternal circulation in this fashion (not enough to produce a positive Kleihauer-Betke test for fetal-maternal hemorrhage). The fetal cells can be sorted out and analyzed by a variety of techniques to look for particular DNA sequences, but without the risks that invasive procedures inherently have. This technique may also make use of the phenomenon of free floating fetal DNA gaining access to maternal circulation by DNA release following apoptosis of placental tissue where the placental tissue in question contains DNA of the same genotype as the fetus. The free floating DNA found in maternal plasma has been shown to contain fetal DNA in proportions as high as 30-40% fetal DNA.

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[0541] In an embodiment, blood may be drawn from a pregnant woman. Research has shown that maternal blood may contain a small amount of free floating DNA from the fetus, in addition to free floating DNA of maternal origin. In addition, there also may be enucleated fetal blood cells comprising DNA of fetal origin, in addition to many blood cells of maternal origin, which typically do not contain nuclear DNA. There are many methods know in the art to isolate fetal DNA, or create fractions enriched in fetal DNA. For example, chromatography has been show to create certain fractions that are enriched in fetal DNA.

[0542] Once the sample of maternal blood, plasma, or other fluid, drawn in a relatively non-invasive manner, and that contains an amount of fetal DNA, either cellular or free floating, either enriched in its proportion to the maternal DNA, or in its original ratio, is in hand, one may genotype the DNA found in said sample. In some embodiments, the blood may be drawn using a needle to withdraw blood from a vein, for example, the basilica vein. The method described herein can be used to determine genotypic data of the fetus. For example, it can be used to determine the ploidy state at one or more chromosomes, it can be used to determine the identity of one or a set of SNPs, including insertions, deletions, and translocations. It can be used to determine one or more haplotypes, including the parent of origin of one or more genotypic features.

[0543] Note that this method will work with any nucleic acids that can be used for any genotyping and/or sequencing methods, such as the ILLUMINA INFINIUM ARRAY platform, AFFYMETRIX GENECHIP, ILLUMINA GENOME ANALYZER, or LIFE TECHNOLGIES' SOLID SYSTEM. This includes extracted free-floating DNA from plasma or amplifications (*e.g.* whole genome amplification, PCR) of the same; genomic DNA from other cell types (*e.g.* human lymphocytes from whole blood) or amplifications of the same. For preparation of the DNA, any extraction or purification method that generates genomic DNA suitable for the one of these platforms will work as well. This method could work equally well with samples of RNA. In an embodiment, storage of the samples may be done in a way that will minimize degradation (*e.g.* below freezing, at about -20 C, or at a lower temperature).

Parental Support

[0544] Some embodiments may be used in combination with the PARENTAL SUPPORT™ (PS) method, embodiments of which are described in U.S. Application No. 11/603,406 (US

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Publication No.: 20070184467), U.S. Application No. 12/076,348 (US Publication No.: 20080243398), U.S. Application 13/110,685, PCT Application PCT/US09/52730 (PCT Publication No.: WO/2010/017214), and PCT Application No. PCT/US10/050824 (PCT Publication No.: WO/2011/041485) which are incorporated herein by reference in their entirety. PARENTAL SUPPORT™ is an informatics based approach that can be used to analyze genetic data. In some embodiments, the methods disclosed herein may be considered as part of the PARENTAL SUPPORT™ method. In some embodiments, The PARENTAL SUPPORT ™ method is a collection of methods that may be used to determine the genetic data of a target individual, with high accuracy, of one or a small number of cells from that individual, or of a mixture of DNA consisting of DNA from the target individual and DNA from one or a plurality of other individuals, specifically to determine disease-related alleles, other alleles of interest, and/or the ploidy state of one or a plurality of chromosomes in the target individual. PARENTAL SUPPORT™ may refer to any of these methods. PARENTAL SUPPORT™ is an example of an informatics based method. Exemplary embodiments of the PARENTAL SUPPORT™ method are illustrated in FIGs. 29-31G and described in Example 19.

[0545] The PARENTAL SUPPORT™ method makes use of known parental genetic data, *i.e.* haplotypic and/or diploid genetic data of the mother and/or the father, together with the knowledge of the mechanism of meiosis and the imperfect measurement of the target DNA, and possibly of one or more related individuals, along with population based crossover frequencies, in order to reconstruct, *in silico*, the genotype at a plurality of alleles, and/or the ploidy state of an embryo or of any target cell(s), and the target DNA at the location of key loci with a high degree of confidence. The PARENTAL SUPPORT™ method can reconstruct not only single nucleotide polymorphisms (SNPs) that were measured poorly, but also insertions and deletions, and SNPs or whole regions of DNA that were not measured at all. Furthermore, the PARENTAL SUPPORT™ method can both measure multiple disease-linked loci as well as screen for aneuploidy, from a single cell. In some embodiments, the PARENTAL SUPPORT™ method may be used to characterize one or more cells from embryos biopsied during an IVF cycle to determine the genetic condition of the one or more cells.

[0546] The PARENTAL SUPPORT™ method allows the cleaning of noisy genetic data. This may be done by inferring the correct genetic alleles in the target genome (embryo) using the genotype of related individuals (parents) as a reference. PARENTAL SUPPORT™ may be particularly relevant where only a small quantity of genetic material is available (*e.g.* PGD) and

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where direct measurements of the genotypes are inherently noisy due to the limited amounts of genetic material. PARENTAL SUPPORT™ may be particularly relevant where only a small fraction of the genetic material available is from the target individual (*e.g.* NPD) and where direct measurements of the genotypes are inherently noisy due to the contaminating DNA signal from another individual. The PARENTAL SUPPORT™ method is able to reconstruct highly accurate ordered diploid allele sequences on the embryo, together with copy number of chromosomes segments, even though the conventional, unordered diploid measurements may be characterized by high rates of allele dropouts, drop-ins, variable amplification biases and other errors. The method may employ both an underlying genetic model and an underlying model of measurement error. The genetic model may determine both allele probabilities at each SNP and crossover probabilities between SNPs. Allele probabilities may be modeled at each SNP based on data obtained from the parents and model crossover probabilities between SNPs based on data obtained from the HapMap database, as developed by the International HapMap Project. Given the proper underlying genetic model and measurement error model, *maximum a posteriori* (MAP) estimation may be used, with modifications for computationally efficiency, to estimate the correct, ordered allele values at each SNP in the embryo.

[0547] The techniques outlined above, in some cases, are able to determine the genotype of an individual given a very small amount of DNA originating from that individual. This could be the DNA from one or a small number of cells, or it could be from the small amount of fetal DNA found in maternal blood.

Hypotheses

[0548] In the context of this disclosure, a hypothesis refers to a possible genetic state. It may refer to a possible ploidy state. It may refer to a possible allelic state. A set of hypotheses may refer to a set of possible genetic states, a set of possible allelic states, a set of possible ploidy states, or combinations thereof. In some embodiments, a set of hypotheses may be designed such that one hypothesis from the set will correspond to the actual genetic state of any given individual. In some embodiments, a set of hypotheses may be designed such that every possible genetic state may be described by at least one hypothesis from the set. In some embodiments of the present disclosure, one aspect of a method is to determine which hypothesis corresponds to the actual genetic state of the individual in question.

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[0549] In another embodiment of the present disclosure, one step involves creating a hypothesis. In some embodiments it may be a copy number hypothesis. In some embodiments it may involve a hypothesis concerning which segments of a chromosome from each of the related individuals correspond genetically to which segments, if any, of the other related individuals. Creating a hypothesis may refer to the act of setting the limits of the variables such that the entire set of possible genetic states that are under consideration are encompassed by those variables.

[0550] A "copy number hypothesis," also called a "ploidy hypothesis," or a "ploidy state hypothesis," may refer to a hypothesis concerning a possible ploidy state for a given chromosome copy, chromosome type, or section of a chromosome, in the target individual. It may also refer to the ploidy state at more than one of the chromosome types in the individual. A set of copy number hypotheses may refer to a set of hypotheses where each hypothesis corresponds to a different possible ploidy state in an individual. A set of hypotheses may concern a set of possible ploidy states, a set of possible parental haplotypes contributions, a set of possible fetal DNA percentages in the mixed sample, or combinations thereof. In some embodiments, the copy number hypotheses include all fetuses in a multiple pregnancy being euploid, all fetuses in a multiple pregnancy being aneuploid (such as any of the aneuploidies disclosed herein), and/or one or more fetuses in a multiple pregnancy being euploid and one or more fetuses in a multiple pregnancy being aneuploidy. In some embodiments, the copy number hypotheses include identical twins (also referred to as monozygotic twins) or fraternal twins (also referred to as dizygotic twins). In some embodiments, the copy number hypotheses include a molar pregnancy, such as a complete or partial molar pregnancy.

[0551] A normal individual contains one of each chromosome type from each parent. However, due to errors in meiosis and mitosis, it is possible for an individual to have 0, 1, 2, or more of a given chromosome type from each parent. In practice, it is rare to see more that two of a given chromosomes from a parent. In this disclosure, some embodiments only consider the possible hypotheses where 0, 1, or 2 copies of a given chromosome come from a parent; it is a trivial extension to consider more or less possible copies originating from a parent. In some embodiments, for a given chromosome, there are nine possible hypotheses: the three possible hypothesis concerning 0, 1, or 2 chromosomes of maternal origin, multiplied by the three possible hypotheses concerning 0, 1, or 2 chromosomes of paternal origin. Let (m,f) refer to the hypothesis where m is the number of a given chromosome inherited from the mother, and f is the number of a given chromosome inherited from the father. Therefore, the nine hypotheses are

(0,0), (0,1), (0,2), (1,0), (1,1), (1,2), (2,0), (2,1), and (2,2). These may also be written as H_{00}, H_{01}, H_{02}, H_{10}, H_{12}, H_{20}, H_{21}, and H_{22}. The different hypotheses correspond to different ploidy states. For example, (1,1) refers to a normal disomic chromosome; (2,1) refers to a maternal trisomy, and (0,1) refers to a paternal monosomy. In some embodiments, the case where two chromosomes are inherited from one parent and one chromosome is inherited from the other parent may be further differentiated into two cases: one where the two chromosomes are identical (matched copy error), and one where the two chromosomes are homologous but not identical (unmatched copy error). In these embodiments, there are sixteen possible hypotheses. It should be understood that it is possible to use other sets of hypotheses, and a different number of hypotheses.

[0552] In some embodiments of the present disclosure, the ploidy hypothesis refers to a hypothesis concerning which chromosome from other related individuals correspond to a chromosome found in the target individual's genome. In some embodiments, a key to the method is the fact that related individuals can be expected to share haplotype blocks, and using measured genetic data from related individuals, along with a knowledge of which haplotype blocks match between the target individual and the related individual, it is possible to infer the correct genetic data for a target individual with higher confidence than using the target individual's genetic measurements alone. As such, in some embodiments, the ploidy hypothesis may concern not only the number of chromosomes, but also which chromosomes in related individuals are identical, or nearly identical, with one or more chromosomes in the target individual.

[0553] Once the set of hypotheses have been defined, when the algorithms operate on the input genetic data, they may output a determined statistical probability for each of the hypotheses under consideration. The probabilities of the various hypotheses may be determined by mathematically calculating, for each of the various hypotheses, the value that the probability equals, as stated by one or more of the expert techniques, algorithms, and/or methods described elsewhere in this disclosure, using the relevant genetic data as input.

[0554] Once the probabilities of the different hypotheses are estimated, as determined by a plurality of techniques, they may be combined. This may entail, for each hypothesis, multiplying the probabilities as determined by each technique. The product of the probabilities of the hypotheses may be normalized. Note that one ploidy hypothesis refers to one possible ploidy state for a chromosome.

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[0555] The process of "combining probabilities," also called "combining hypotheses," or combining the results of expert techniques, is a concept that should be familiar to one skilled in the art of linear algebra. One possible way to combine probabilities is as follows: When an expert technique is used to evaluate a set of hypotheses given a set of genetic data, the output of the method is a set of probabilities that are associated, in a one-to-one fashion, with each hypothesis in the set of hypotheses. When a set of probabilities that were determined by a first expert technique, each of which are associated with one of the hypotheses in the set, are combined with a set of probabilities that were determined by a second expert technique, each of which are associated with the same set of hypotheses, then the two sets of probabilities are multiplied. This means that, for each hypothesis in the set, the two probabilities that are associated with that hypothesis, as determined by the two expert methods, are multiplied together, and the corresponding product is the output probability. This process may be expanded to any number of expert techniques. If only one expert technique is used, then the output probabilities are the same as the input probabilities. If more than two expert techniques are used, then the relevant probabilities may be multiplied at the same time. The products may be normalized so that the probabilities of the hypotheses in the set of hypotheses sum to 100%.

[0556] In some embodiments, if the combined probabilities for a given hypothesis are greater than the combined probabilities for any of the other hypotheses, then it may be considered that that hypothesis is determined to be the most likely. In some embodiments, a hypothesis may be determined to be the most likely, and the ploidy state, or other genetic state, may be called if the normalized probability is greater than a threshold. In an embodiment, this may mean that the number and identity of the chromosomes that are associated with that hypothesis may be called as the ploidy state. In an embodiment, this may mean that the identity of the alleles that are associated with that hypothesis may be called as the allelic state. In some embodiments, the threshold may be between about 50% and about 80%. In some embodiments the threshold may be between about 80% and about 90%. In some embodiments the threshold may be between about 90% and about 95%. In some embodiments the threshold may be between about 95% and about 99%. In some embodiments the threshold may be between about 99% and about 99.9%. In some embodiments the threshold may be above about 99.9%.

Parental Contexts

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[0557] The parental context refers to the genetic state of a given allele, on each of the two relevant chromosomes for one or both of the two parents of the target. Note that in an embodiment, the parental context does not refer to the allelic state of the target, rather, it refers to the allelic state of the parents. The parental context for a given SNP may consist of four base pairs, two paternal and two maternal; they may be the same or different from one another. It is typically written as "$m_1m_2|f_1f_2$," where m_1 and m_2 are the genetic state of the given SNP on the two maternal chromosomes, and f_1 and f_2 are the genetic state of the given SNP on the two paternal chromosomes. In some embodiments, the parental context may be written as "$f_1f_2|m_1m_2$." Note that subscripts "1" and "2" refer to the genotype, at the given allele, of the first and second chromosome; also note that the choice of which chromosome is labeled "1" and which is labeled "2" is arbitrary.

[0558] Note that in this disclosure, A and B are often used to generically represent base pair identities; A or B could equally well represent C (cytosine), G (guanine), A (adenine) or T (thymine). For example, if, at a given SNP based allele, the mother's genotype was T at that SNP on one chromosome, and G at that SNP on the homologous chromosome, and the father's genotype at that allele is G at that SNP on both of the homologous chromosomes, one may say that the target individual's allele has the parental context of AB|BB; it could also be said that the allele has the parental context of AB|AA. Note that, in theory, any of the four possible nucleotides could occur at a given allele, and thus it is possible, for example, for the mother to have a genotype of AT, and the father to have a genotype of GC at a given allele. However, empirical data indicate that in most cases only two of the four possible base pairs are observed at a given allele. It is possible, for example when using single tandem repeats, to have more than two parental, more than four and even more than ten contexts. In this disclosure the discussion assumes that only two possible base pairs will be observed at a given allele, although the embodiments disclosed herein could be modified to take into account the cases where this assumption does not hold.

[0559] A "parental context" may refer to a set or subset of target SNPs that have the same parental context. For example, if one were to measure 1000 alleles on a given chromosome on a target individual, then the context AA|BB could refer to the set of all alleles in the group of 1,000 alleles where the genotype of the mother of the target was homozygous, and the genotype of the father of the target is homozygous, but where the maternal genotype and the paternal genotype are dissimilar at that locus. If the parental data is not phased, and thus AB = BA, then there are

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nine possible parental contexts: AA|AA, AA|AB, AA|BB, AB|AA, AB|AB, AB|BB, BB|AA, BB|AB, and BB|BB. If the parental data is phased, and thus AB $\neq$ BA, then there are sixteen different possible parental contexts: AA|AA, AA|AB, AA|BA, AA|BB, AB|AA, AB|AB, AB|BA, AB|BB, BA|AA, BA|AB, BA|BA, BA|BB, BB|AA, BB|AB, BB|BA, and BB|BB. Every SNP allele on a chromosome, excluding some SNPs on the sex chromosomes, has one of these parental contexts. The set of SNPs wherein the parental context for one parent is heterozygous may be referred to as the heterozygous context.

Use of Parental Contexts in NPD

[0560] Non-invasive prenatal diagnosis is an important technique that can be used to determine the genetic state of a fetus from genetic material that is obtained in a non-invasive manner, for example from a blood draw on the pregnant mother. The blood could be separated and the plasma isolated, followed by isolation of the plasma DNA. Size selection could be used to isolate the DNA of the appropriate length. The DNA may be preferentially enriched at a set of loci. This DNA can then be measured by a number of means, such as by hybridizing to a genotyping array and measuring the fluorescence, or by sequencing on a high throughput sequencer.

[0561] When sequencing is used for ploidy calling of a fetus in the context of non-invasive prenatal diagnosis, there are a number of ways to use the sequence data. The most common way one could use the sequence data is to simply count the number of reads that map to a given chromosome. For example, imagine if you are trying to determine the ploidy state of chromosome 21 on the fetus. Further imagine that the DNA in the sample is comprised of 10% DNA of fetal origin, and 90% DNA of maternal origin. In this case, you could look at the average number of reads on a chromosome which can be expected to be disomic, for example chromosome 3, and compare that to the number of read on chromosome 21, where the reads are adjusted for the number of base pairs on that chromosome that are part of a unique sequence. If the fetus were euploid, one would expect the amount of DNA per unit of genome to be about equal at all locations (subject to stochastic variations). On the other hand, if the fetus were trisomic at chromosome 21, then one would expect there to be more slightly more DNA per genetic unit from chromosome 21 than the other locations on the genome. Specifically one would expect there to be about 5% more DNA from chromosome 21 in the mixture. When sequencing is used to measure the DNA, one would expect about 5% more uniquely mappable

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reads from chromosome 21 per unique segment than from the other chromosomes. One could use the observation of an amount of DNA from a particular chromosome that is higher than a certain threshold, when adjusted for the number of sequences that are uniquely mappable to that chromosome, as the basis for an aneuploidy diagnosis. Another method that may be used to detect aneuploidy is similar to that above, except that parental contexts could be taken into account.

[0562] When considering which alleles to target, one may consider the likelihood that some parental contexts are likely to be more informative than others. For example, AA|BB and the symmetric context BB|AA are the most informative contexts, because the fetus is known to carry an allele that is different from the mother. For reasons of symmetry, both AA|BB and BB|AA contexts may be referred to as AA|BB. Another set of informative parental contexts are AA|AB and BB|AB, because in these cases the fetus has a 50% chance of carrying an allele that the mother does not have. For reasons of symmetry, both AA|AB and BB|AB contexts may be referred to as AA|AB. A third set of informative parental contexts are AB|AA and AB|BB, because in these cases the fetus is carrying a known paternal allele, and that allele is also present in the maternal genome. For reasons of symmetry, both AB|AA and AB|BB contexts may be referred to as AB|AA. A fourth parental context is AB|AB where the fetus has an unknown allelic state, and whatever the allelic state, it is one in which the mother has the same alleles. The fifth parental context is AA|AA, where the mother and father are heterozygous.

Different Implementations of the Presently Disclosed Embodiments

[0563] Methods are disclosed herein for determining the ploidy state of a target individual. The target individual may be a blastomere, an embryo, or a fetus. In some embodiments of the present disclosure, a method for determining the ploidy state of one or more chromosome in a target individual may include any of the steps described in this document, and combinations thereof:

[0564] In some embodiments the source of the genetic material to be used in determining the genetic state of the fetus may be fetal cells, such as nucleated fetal red blood cells, isolated from the maternal blood. The method may involve obtaining a blood sample from the pregnant mother. The method may involve isolating a fetal red blood cell using visual techniques, based on the idea that a certain combination of colors are uniquely associated with nucleated red blood cell, and a similar combination of colors is not associated with any other present cell in the

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maternal blood. The combination of colors associated with the nucleated red blood cells may include the red color of the hemoglobin around the nucleus, which color may be made more distinct by staining, and the color of the nuclear material which can be stained, for example, blue. By isolating the cells from maternal blood and spreading them over a slide, and then identifying those points at which one sees both red (from the Hemoglobin) and blue (from the nuclear material) one may be able to identify the location of nucleated red blood cells. One may then extract those nucleated red blood cells using a micromanipulator, use genotyping and/or sequencing techniques to measure aspects of the genotype of the genetic material in those cells.

[0565] In an embodiment, one may stain the nucleated red blood cell with a die that only fluoresces in the presence of fetal hemoglobin and not maternal hemoglobin, and so remove the ambiguity between whether a nucleated red blood cell is derived from the mother or the fetus. Some embodiments of the present disclosure may involve staining or otherwise marking nuclear material. Some embodiments of the present disclosure may involve specifically marking fetal nuclear material using fetal cell specific antibodies.

[0566] There are many other ways to isolate fetal cells from maternal blood, or fetal DNA from maternal blood, or to enrich samples of fetal genetic material in the presence of maternal genetic material. Some of these methods are listed here, but this is not intended to be an exhaustive list. Some appropriate techniques are listed here for convenience: using fluorescently or otherwise tagged antibodies, size exclusion chromatography, magnetically or otherwise labeled affinity tags, epigenetic differences, such as differential methylation between the maternal and fetal cells at specific alleles, density gradient centrifugation succeeded by CD45/14 depletion and CD71-positive selection from CD45/14 negative-cells, single or double Percoll gradients with different osmolalities, or galactose specific lectin method.

[0567] In an embodiment of the present disclosure, the target individual is a fetus, and the different genotype measurements are made on a plurality of DNA samples from the fetus. In some embodiments of the present disclosure, the fetal DNA samples are from isolated fetal cells where the fetal cells may be mixed with maternal cells. In some embodiments of the present disclosure, the fetal DNA samples are from free floating fetal DNA, where the fetal DNA may be mixed with free floating maternal DNA. In some embodiments, the fetal DNA samples may be derived from maternal plasma or maternal blood that contains a mixture of maternal DNA and fetal DNA. In some embodiments, the fetal DNA may be mixed with maternal DNA in maternal:fetal ratios ranging from 99.9:0.1% to 99:1%; 99:1% to 90:10%; 90:10% to 80:20%;

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80:20% to 70:30%; 70:30% to 50:50%; 50:50% to 10:90%; or 10:90% to 1:99%; 1:99% to 0.1:99.9%.

[0568] The genetic data of the target individual and/or of the related individual can be transformed from a molecular state to an electronic state by measuring the appropriate genetic material using tools and or techniques taken from a group including, but not limited to: genotyping microarrays, and high throughput sequencing. Some high throughput sequencing methods include Sanger DNA sequencing, pyrosequencing, the ILLUMINA SOLEXA platform, ILLUMINA's GENOME ANALYZER, or APPLIED BIOSYSTEM's 454 sequencing platform, HELICOS's TRUE SINGLE MOLECULE SEQUENCING platform, HALCYON MOLECULAR's electron microscope sequencing method, or any other sequencing method,. All of these methods physically transform the genetic data stored in a sample of DNA into a set of genetic data that is typically stored in a memory device in route to being processed.

[0569] A relevant individual's genetic data may be measured by analyzing substances taken from a group including, but not limited to: the individual's bulk diploid tissue, one or more diploid cells from the individual, one or more haploid cells from the individual, one or more blastomeres from the target individual, extra-cellular genetic material found on the individual, extra-cellular genetic material from the individual found in maternal blood, cells from the individual found in maternal blood, one or more embryos created from (a) gamete(s) from the related individual, one or more blastomeres taken from such an embryo, extra-cellular genetic material found on the related individual, genetic material known to have originated from the related individual, and combinations thereof.

[0570] In some embodiments, a set of at least one ploidy state hypothesis may be created for each of the chromosomes types of interest of the target individual. Each of the ploidy state hypotheses may refer to one possible ploidy state of the chromosome or chromosome segment of the target individual. The set of hypotheses may include some or all of the possible ploidy states that the chromosome of the target individual may be expected to have. Some of the possible ploidy states may include nullsomy, monosomy, disomy, uniparental disomy, euploidy, trisomy, matching trisomy, unmatching trisomy, maternal trisomy, paternal trisomy, tetrasomy, balanced (2:2) tetrasomy, unbalanced (3:1) tetrasomy, pentasomy, hexasomy, other aneuploidy, and combinations thereof. Any of these aneuploidy states may be mixed or partial aneuploidy such as unbalanced translocations, balanced translocations, Robertsonian translocations, recombinations, deletions, insertions, crossovers, and combinations thereof.

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[0571] In some embodiments, the knowledge of the determined ploidy state may be used to make a clinical decision. This knowledge, typically stored as a physical arrangement of matter in a memory device, may then be transformed into a report. The report may then be acted upon. For example, the clinical decision may be to terminate the pregnancy; alternately, the clinical decision may be to continue the pregnancy. In some embodiments the clinical decision may involve an intervention designed to decrease the severity of the phenotypic presentation of a genetic disorder, or a decision to take relevant steps to prepare for a special needs child.

[0572] In an embodiment of the present disclosure, any of the methods described herein may be modified to allow for multiple targets to come from same target individual, for example, multiple blood draws from the same pregnant mother. This may improve the accuracy of the model, as multiple genetic measurements may provide more data with which the target genotype may be determined. In an embodiment, one set of target genetic data served as the primary data which was reported, and the other served as data to double-check the primary target genetic data. In an embodiment, a plurality of sets of genetic data, each measured from genetic material taken from the target individual, are considered in parallel, and thus both sets of target genetic data serve to help determine which sections of parental genetic data, measured with high accuracy, composes the fetal genome.

[0573] In an embodiment, the method may be used for the purpose of paternity testing. For example, given the SNP-based genotypic information from the mother, and from a man who may or may not be the genetic father, and the measured genotypic information from the mixed sample, it is possible to determine if the genotypic information of the male indeed represents that actual genetic father of the gestating fetus. A simple way to do this is to simply look at the contexts where the mother is AA, and the possible father is AB or BB. In these cases, one may expect to see the father contribution half (AA|AB) or all (AA|BB) of the time, respectively. Taking into account the expected ADO, it is straightforward to determine whether or not the fetal SNPs that are observed are correlated with those of the possible father.

[0574] One embodiment of the present disclosure could be as follows: a pregnant woman wants to know if her fetus is afflicted with Down Syndrome, and/or if it will suffer from Cystic Fibrosis, and she does not wish to bear a child that is afflicted with either of these conditions. A doctor takes her blood, and stains the hemoglobin with one marker so that it appears clearly red, and stains nuclear material with another marker so that it appears clearly blue. Knowing that maternal red blood cells are typically anuclear, while a high proportion of fetal cells contain a

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nucleus, the doctor is able to visually isolate a number of nucleated red blood cells by identifying those cells that show both a red and blue color. The doctor picks up these cells off the slide with a micromanipulator and sends them to a lab which amplifies and genotypes ten individual cells. By using the genetic measurements, the PARENTAL SUPPORT™ method is able to determine that six of the ten cells are maternal blood cells, and four of the ten cells are fetal cells. If a child has already been born to a pregnant mother, PARENTAL SUPPORT™ can also be used to determine that the fetal cells are distinct from the cells of the born child by making reliable allele calls on the fetal cells and showing that they are dissimilar to those of the born child. Note that this method is similar in concept to the paternal testing embodiment of the present disclosure. The genetic data measured from the fetal cells may be of very poor quality, comprising many allele drop outs, due to the difficulty of genotyping single cells. The clinician is able to use the measured fetal DNA along with the reliable DNA measurements of the parents to infer aspects of the genome of the fetus with high accuracy using PARENTAL SUPPORT™, thereby transforming the genetic data contained on genetic material from the fetus into the predicted genetic state of the fetus, stored on a computer. The clinician is able to determine both the ploidy state of the fetus, and the presence or absence of a plurality of disease-linked genes of interest. It turns out that the fetus is euploid, and is not a carrier for cystic fibrosis, and the mother decides to continue the pregnancy.

[0575] In an embodiment of the present disclosure, a pregnant mother would like to determine if her fetus is afflicted with any whole chromosomal abnormalities. She goes to her doctor, and gives a sample of her blood, and she and her husband gives samples of their own DNA from cheek swabs. A laboratory researcher genotypes the parental DNA using the MDA protocol to amplify the parental DNA, and ILLUMINA INFINIUM arrays to measure the genetic data of the parents at a large number of SNPs. The researcher then spins down the blood, takes the plasma, and isolates a sample of free-floating DNA using size exclusion chromatography. Alternately, the researcher uses one or more fluorescent antibodies, such as one that is specific to fetal hemoglobin to isolate a nucleated fetal red blood cell. The researcher then takes the isolated or enriched fetal genetic material and amplifies it using a library of 70-mer oligonucleotides appropriately designed such that two ends of each oligonucleotide corresponded to the flanking sequences on either side of a target allele. Upon addition of a polymerase, ligase, and the appropriate reagents, the oligonucleotides underwent gap-filling circularization, capturing the desired allele. An exonuclease was added, heat-inactivated, and the

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