(19) **United States**

(12) **Patent Application Publication** (10) Pub. No.: **US 2010/0136529 A1**

Shoemaker et al. (43) **Pub. Date:** **Jun. 3, 2010**

(54) **RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS**

(76) Inventors: **Daniel Shoemaker**, San Diego, CA (US); **Martin Fuchs**, Uxbridge, MA (US); **Neil X. Krueger**, Roslindale, MA (US); **Mehmet Toner**, Wellesley Hills, MA (US); **Darren Gray**, Brookline, MA (US); **Ravi Kapur**, Stoughton, MA (US); **Zihua Wang**, Newton, MA (US)

Correspondence Address:
FISH & RICHARDSON PC
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022 (US)

(21) Appl. No.: **12/230,628**

(22) Filed: **Sep. 2, 2008**

Related U.S. Application Data

(63) Continuation of application No. 11/763,421, filed on Jun. 14, 2007.

(60) Provisional application No. 60/804,819, filed on Jun. 14, 2006.

Publication Classification

(51) **Int. Cl.**
C12Q 1/68	(2006.01)
G01N 33/53	(2006.01)
G06N 5/02	(2006.01)

(52) **U.S. Cl.** **435/6**; 436/94; 706/54

(57) **ABSTRACT**

The present invention provides systems, apparatuses, and methods to detect the presence of fetal cells when mixed with a population of maternal cells in a sample and to test fetal abnormalities, e.g. aneuploidy. The present invention involves labeling regions of genomic DNA in each cell in said mixed sample with different labels wherein each label is specific to each cell and quantifying the labeled regions of genomic DNA from each cell in the mixed sample. More particularly the invention involves quantifying labeled DNA polymorphisms from each cell in the mixed sample.
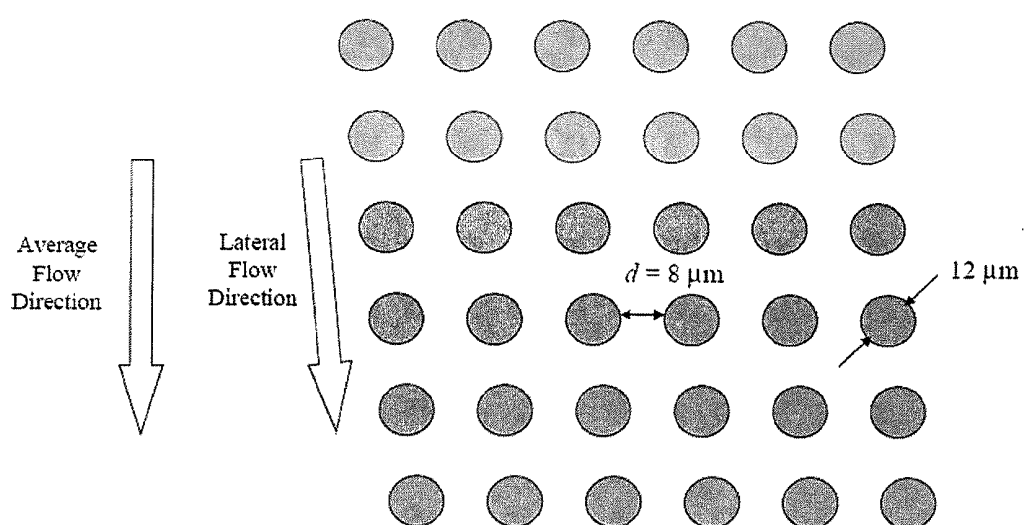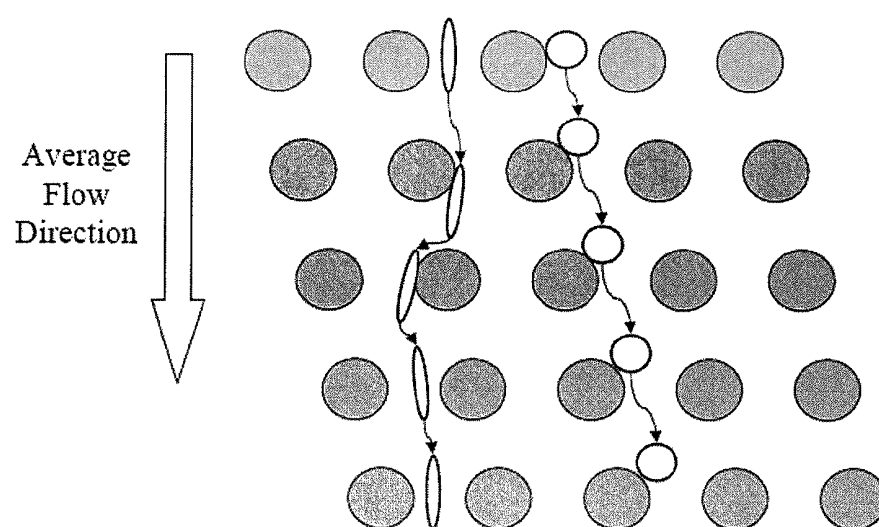
Average Flow Direction

Lateral Flow Direction

$d = 8$ μm

12 μm

Average
Flow
Direction

Lateral
Flow
Direction

$d = 8 \ \mu m$

12 μm

Figure 1A

Average
Flow
Direction

Figure 1B

Average
Flow
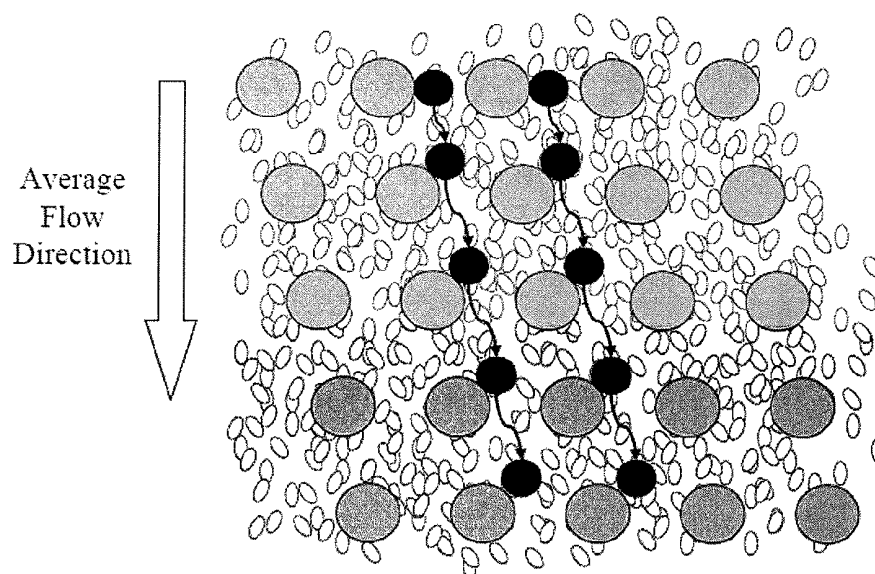Direction

Figure 1C

Average
Flow
Direction
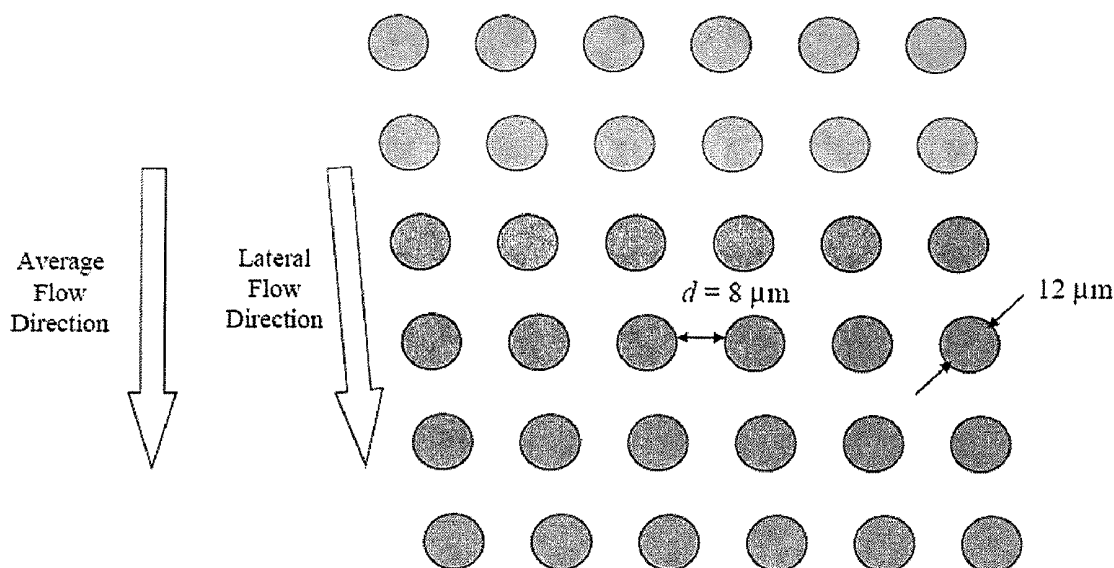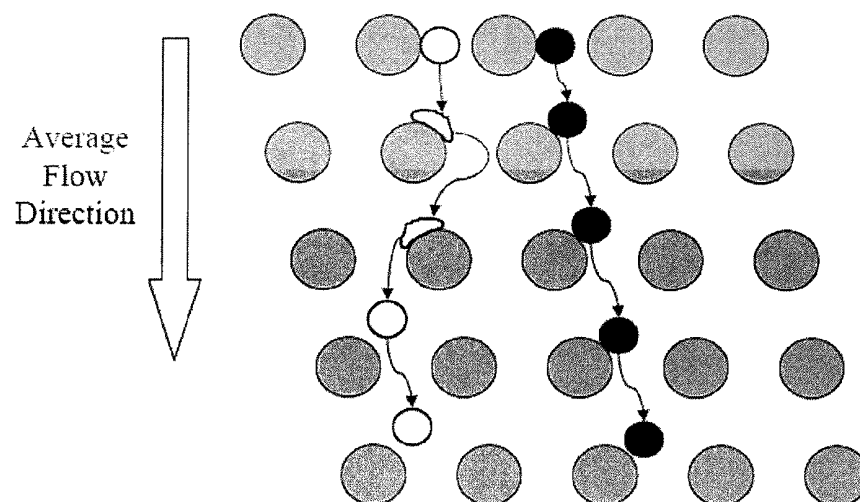
Figure 1D

Microposts and cells
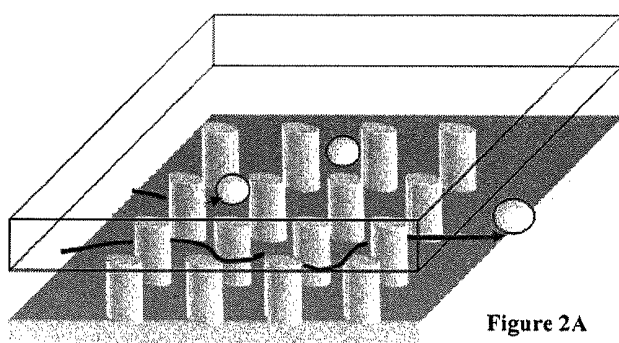
Figure 2A
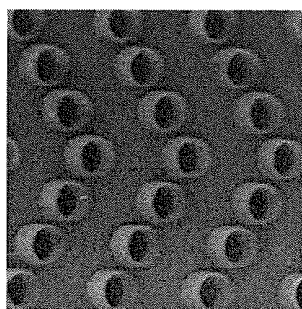
Figure 2B

Antibody
coated
posts
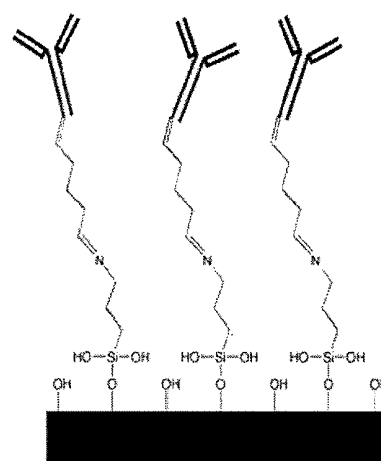
Figure 2C

Figure 3

Figure 4

Obtain sample —— 500

Enrich for rare cells —— 502

Split enriched product —— 504

Amplify highly polymorphic DNA regions —— 506

Tag each polymorphic DNA region —— 508

Pool amplicons —— 510

Determine genotype of amplicons —— 512

Analyze genotyping data —— 514

Identify cells with rare alleles —— 516

NO → Stop

Determine condition based on rare cell polymorphism —— 518
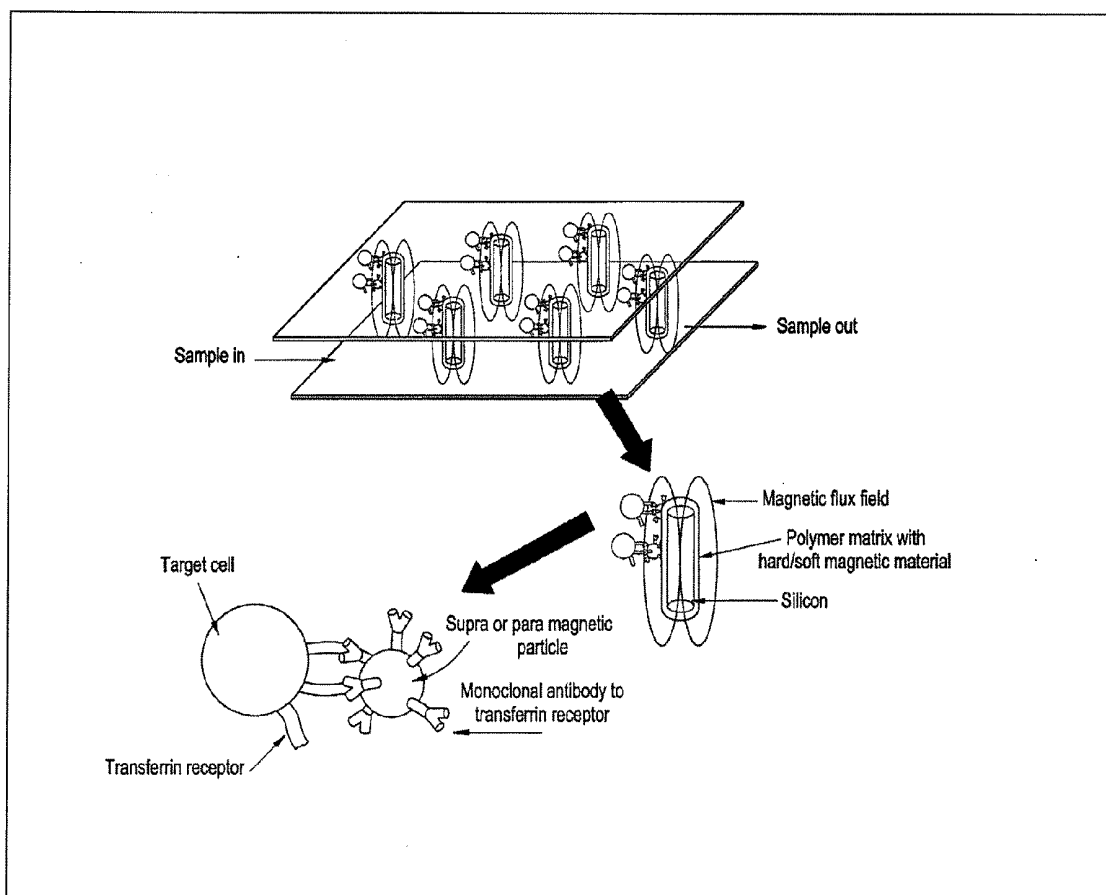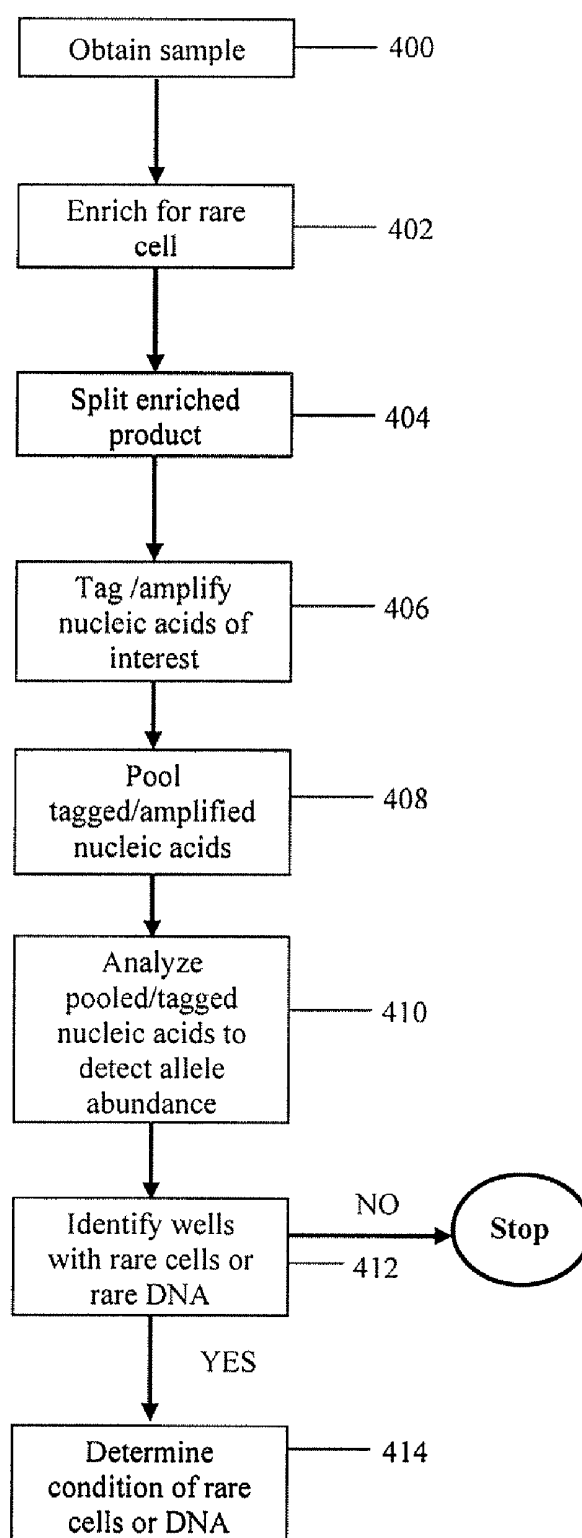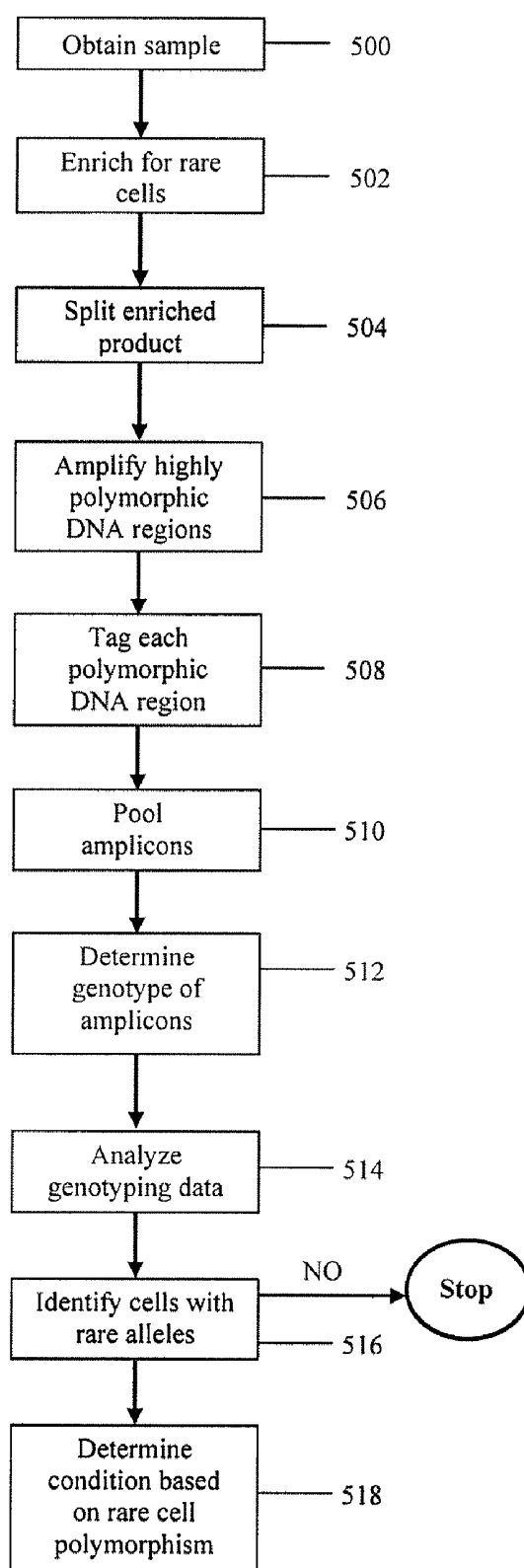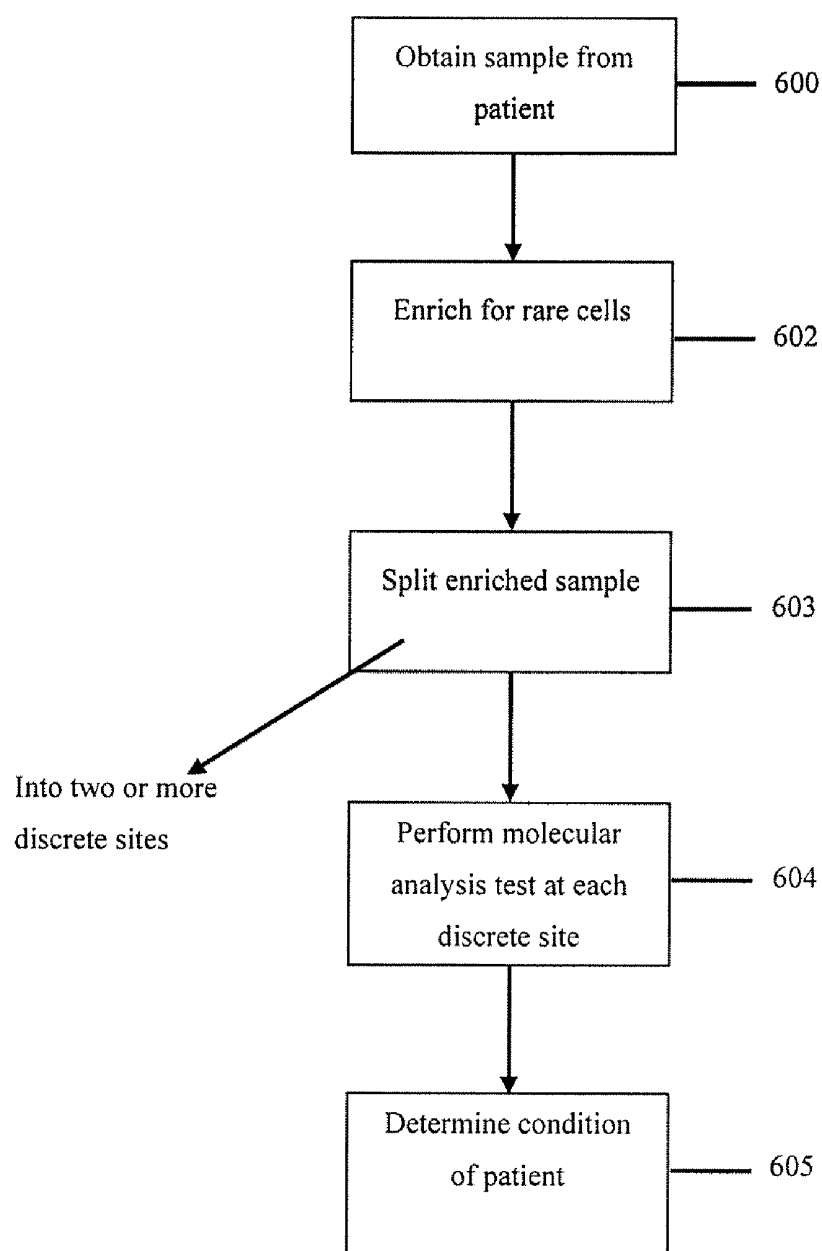
Figure 5

Figure 6
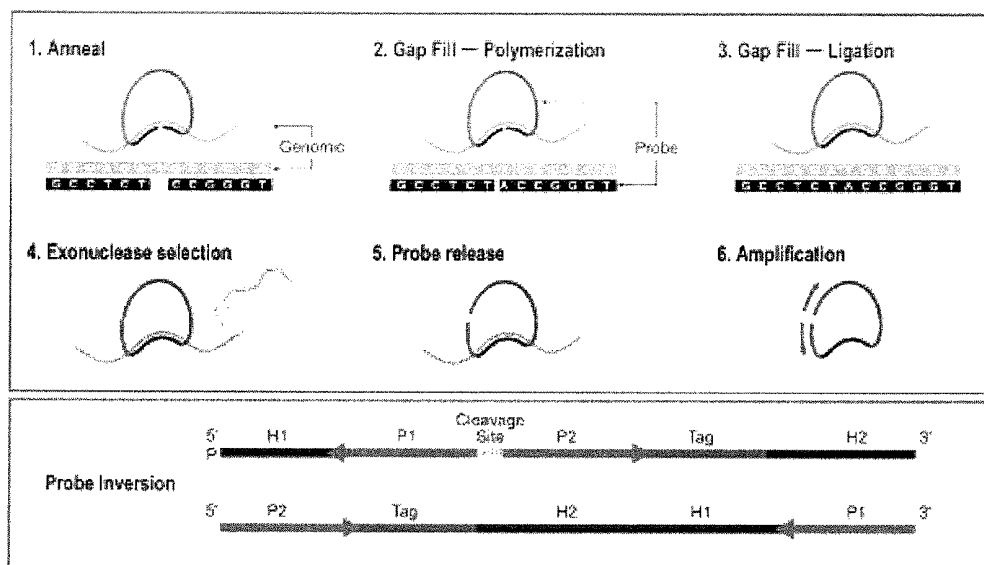
Molecular Inversion Probes are so named because the oligonucleotide probe central to the process undergoes a unimolecular rearrangement from a molecule that cannot be amplified (step 1), into a molecule that can be amplified (step 6). This rearrangement is mediated by hybridization to genomic DNA (step 2) and an enzymatic "gap fill" process that occurs in an allele-specific manner (step 3). The resulting circularized probe can be separated from cross-reacted or unreacted probes by a simple exonuclease reaction (step 4). Figure 1 shows these steps.

Figure 7A

Figure 7B: 40-plex MIP assay performed for each of the 500 wells

Figure 7C

1536 well plate
32 x 48 wells

Current design has 14
outputs. Increase to 16 or
32 outputs.
Dispense into 32 wells
simultaneously.

Figure   8A

Figure 8B

Isometric View
(not illustrative
of actual well
density)

Figure 8C

Cross-section

Figure 9

Figure 10

2AR

A DISINTEGRIN

ACTIVATOR OF THYROID AND RETINOIC ACID RECEPTOR (ACTR)

ADAM 11

ADIPOGENESIS INHIBITORY FACTOR (ADIF)

ALPHA 6 INTEGRIN SUBUNIT

ALPHA V INTEGRIN SUBUNIT

ALPHA-CATENIN

AMPLIFIED IN BREAST CANCER 1 (AIB1)

AMPLIFIED IN BREAST CANCER 3 (AIB3)

AMPLIFIED IN BREAST CANCER 4 (AIB4)

AMYLOID PRECURSOR PROTEIN SECRETASE (APPS)

AP-2 GAMMA
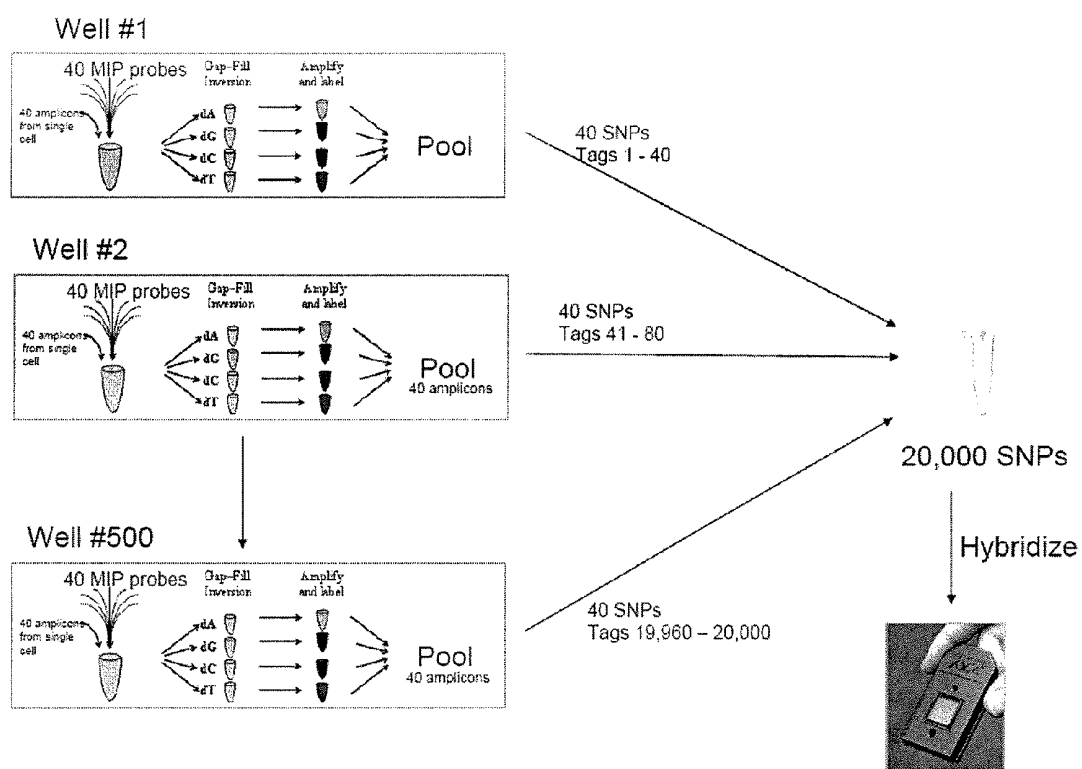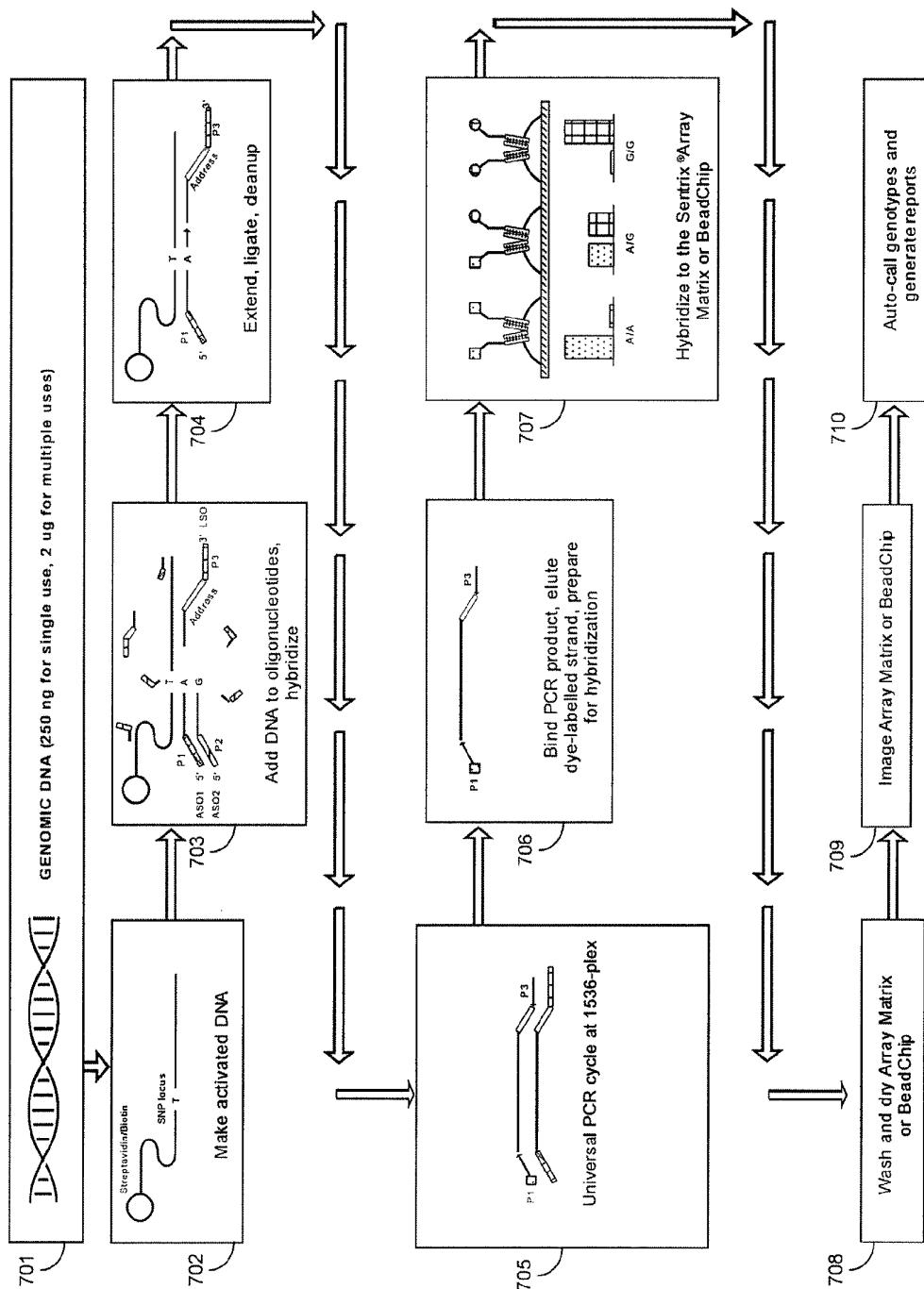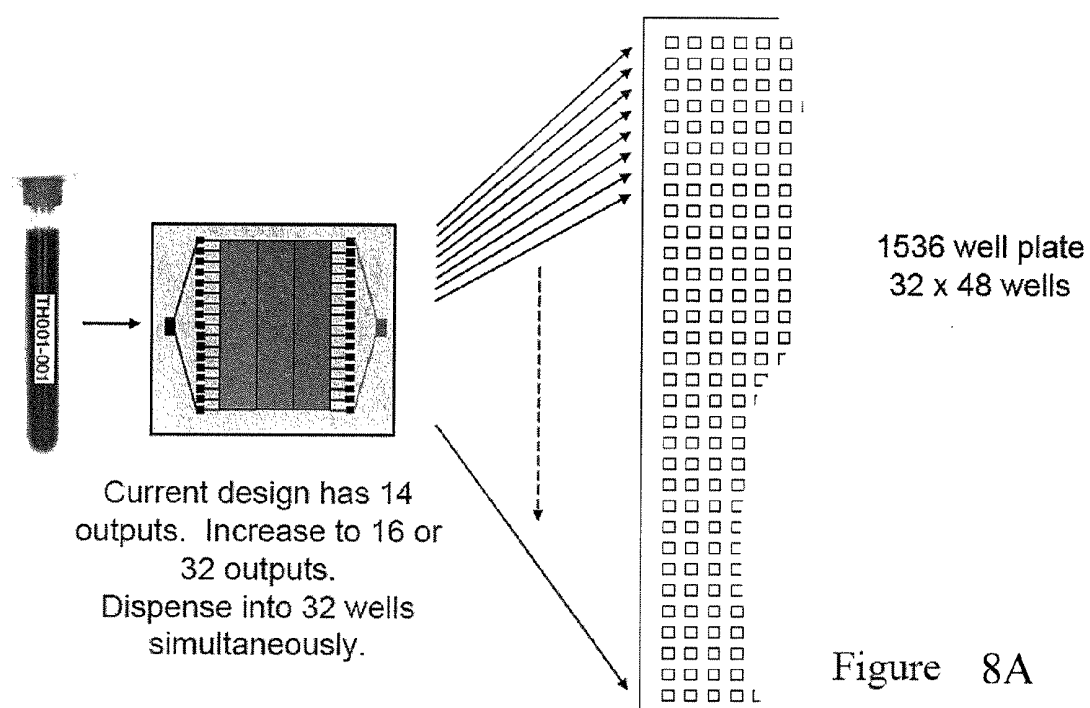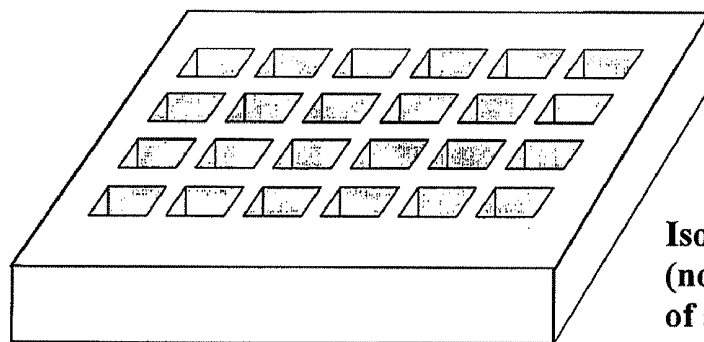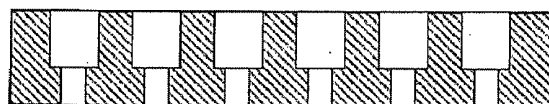
APPS

ATP-BINDING CASSETTE TRANSPORTER (ABCT)

PLACENTA-SPECIFIC (ABCP)

ATP-BINDING CASSETTE SUBFAMILY C MEMBER (ABCC1)

BAG-1

BASIGIN (BSG)

BCEI

B-CELL DIFFERENTIATION FACTOR (BCDF)

B-CELL LEUKEMIA 2 (BCL-2)

B-CELL STIMULATORY FACTOR-2 (BSF-2)

BCL-1

BCL-2-ASSOCIATED X PROTEIN (BAX)

BCRP

BETA 1 INTEGRIN SUBUNIT

BETA 3 INTEGRIN SUBUNIT

BETA 5 INTEGRIN SUBUNIT

BETA-2 INTERFERON

BETA-CATENIN

BETA-CATENIN

BONE SIALOPROTEIN (BSP)

BREAST CANCER ESTROGEN-INDUCIBLE SEQUENCE (BCEI)

BREAST CANCER RESISTANCE PROTEIN (BCRP)

BREAST CANCER TYPE 1 (BRCA1)

BREAST CANCER TYPE 2 (BRCA2)

BREAST CARCINOMA AMPLIFIED SEQUENCE 2 (BCAS2)

CADHERIN

EPITHELIAL CADHERIN-11

CADHERIN-ASSOCIATED PROTEIN

CALCITONIN RECEPTOR (CTR)

CALCIUM PLACENTAL PROTEIN (CAPL)

CALCYCLIN

CALLA

CAM5

CAPL

CARCINOEMBRYONIC ANTIGEN (CEA)

CATENIN

ALPHA 1

CATHEPSIN B

CATHEPSIN D

CATHEPSIN K

CATHEPSIN L2

CATHEPSIN O

CATHEPSIN O1

CATHEPSIN V

CD10

CD146

CD147

CD24

CD29

CD44

CD51

CD54

CD61

CD66e

CD82

CD87

CD9

CEA

CELLULAR RETINOL-BINDING PROTEIN 1 (CRBP1)

c-ERBB-2

CK7

CK8

CK18

CK19

CK20

CLAUDIN-7

c-MET

COLLAGENASE

FIBROBLAST

COLLAGENASE

INTERSTITIAL

COLLAGENASE-3

COMMON ACUTE LYMPHOCYTIC LEUKEMIA ANTIGEN (CALLA)

CONNEXIN 26 (Cx26)

CONNEXIN 43 (Cx43)

CORTACTIN

COX-2

CTLA-8

CTR

CTSD

CYCLIN D1

CYCLOOXYGENASE-2

CYTOKERATIN 18

CYTOKERATIN 19

CYTOKERATIN 8

CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED SERINE ESTERASE 8 (CTLA-8)

DIFFERENTIATION-INHIBITING ACTIVITY (DIA)

DNA AMPLIFIED IN MAMMARY CARCINOMA 1 (DAM1)

DNA TOPOISOMERASE II ALPHA

DR-NM23

E-CADHERIN

EMMPRIN

EMS1

ENDOTHELIAL CELL GROWTH FACTOR (ECGR)

PLATELET-DERIVED (PD-ECGF)

ENKEPHALINASE

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

EPISIALIN

EPITHELIAL MEMBRANE ANTIGEN (EMA)

ER-ALPHA

ERBB2

ERBB4

ER-BETA

ERF-1

ERYTHROID-POTENTIATING ACTIVITY (EPA)

ESR1

ESTROGEN RECEPTOR-ALPHA

ESTROGEN RECEPTOR-BETA

ETS-1

EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER (EMMPRIN)

FIBRONECTIN RECEPTOR BETA POLYPEPTIDE (FNRB)

FIBRONECTIN RECEPTOR BETA SUBUNIT (FNRB)

FLK-1

GA15.3

GA733.2

GALECTIN-3

GAMMA-CATENIN

GAP JUNCTION PROTEIN (26 kDa)

GAP JUNCTION PROTEIN (43 kDa)

GAP JUNCTION PROTEIN ALPHA-1 (GJA1)

GAP JUNCTION PROTEIN BETA-2 (GJB2)

GCP1

GELATINASE A

GELATINASE B

GELATINASE (72 kDa)

GELATINASE (92 kDa)

GLIOSTATIN

GLUCOCORTICOID RECEPTOR INTERACTING PROTEIN 1 (GRIP1)

GLUTATHIONE S-TRANSFERASE p

GM-CSF

GRANULOCYTE CHEMOTACTIC PROTEIN 1 (GCP1)

GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR

GROWTH FACTOR RECEPTOR BOUND-7 (GRB-7)

GSTp

HAP

HEAT-SHOCK COGNATE PROTEIN 70 (HSC70)

HEAT-STABLE ANTIGEN

HEPATOCYTE GROWTH FACTOR (HGF)

HEPATOCYTE GROWTH FACTOR RECEPTOR (HGFR)

HEPATOCYTE-STIMULATING FACTOR III (HSF III)

HER-2

HER2/NEU

HERMES ANTIGEN

HET

HHM

HUMORAL HYPERCALCEMIA OF MALIGNANCY (HHM)

ICERE-1

INT-1

INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1)

INTERFERON-GAMMA-INDUCING FACTOR (IGIF)

INTERLEUKIN-1 ALPHA (IL-1A)

INTERLEUKIN-1 BETA (IL-1B)

INTERLEUKIN-11 (IL-11)

INTERLEUKIN-17 (IL-17)

INTERLEUKIN-18 (IL-18)

INTERLEUKIN-6 (IL-6)

INTERLEUKIN-8 (IL-8)

INVERSELY CORRELATED WITH ESTROGEN RECEPTOR EXPRESSION-1 (ICERE-1)

KAI1

KDR

KERATIN 8

KERATIN 18

KERATIN 19

KISS-1

LEUKEMIA INHIBITORY FACTOR (LIF)

LIF

LOST IN INFLAMMATORY BREAST CANCER (LIBC)

LOT ("LOST ON TRANSFORMATION")

LYMPHOCYTE HOMING RECEPTOR

MACROPHAGE-COLONY STIMULATING FACTOR

MAGE-3

MAMMAGLOBIN

MASPIN

MC56

M-CSF

MDC

MDNCF

MDR

MELANOMA CELL ADHESION MOLECULE (MCAM)

MEMBRANE METALLOENDOPEPTIDASE (MME)

MEMBRANE-ASSOCIATED NEUTRAL ENDOPEPTIDASE (NEP)

CYSTEINE-RICH PROTEIN (MDC)

METASTASIN (MTS-1)

MLN64

MMP1

MMP2

MMP3

MMP7

MMP9

MMP11

MMP13

MMP14

MMP15

MMP16

MMP17

MOESIN

MONOCYTE ARGININE-SERPIN

MONOCYTE-DERIVED NEUTROPHIL CHEMOTACTIC FACTOR

MONOCYTE-DERIVED PLASMINOGEN ACTIVATOR INHIBITOR

MTS-1

MUC-1

MUC18

MUCIN LIKE CANCER ASSOCIATED ANTIGEN (MCA)

MUCIN

MUC-1

MULTIDRUG RESISTANCE PROTEIN 1 (MDR, MDR1)

MULTIDRUG RESISTANCE RELATED PROTEIN-1 (MRP, MRP-1)

N-CADHERIN

NEP

NEU

NEUTRAL ENDOPEPTIDASE

NEUTROPHIL-ACTIVATING PEPTIDE 1 (NAP1)

NM23-H1

NM23-H2

NME1

NME2

NUCLEAR RECEPTOR COACTIVATOR-1 (NCoA-1)

NUCLEAR RECEPTOR COACTIVATOR-2 (NCoA-2)

NUCLEAR RECEPTOR COACTIVATOR-3 (NCoA-3)

NUCLEOSIDE DIPHOSPHATE KINASE A (NDPKA)

NUCLEOSIDE DIPHOSPHATE KINASE B (NDPKB)

ONCOSTATIN M (OSM)

ORNITHINE DECARBOXYLASE (ODC)

OSTEOCLAST DIFFERENTIATION FACTOR (ODF)

OSTEOCLAST DIFFERENTIATION FACTOR RECEPTOR (ODFR)

OSTEONECTIN (OSN, ON)

OSTEOPONTIN (OPN)

OXYTOCIN RECEPTOR (OXTR)

p27/kip1

p300/CBP COINTEGRATOR ASSOCIATE PROTEIN (p/CIP)

p53

p9Ka

PAI-1

PAI-2

PARATHYROID ADENOMATOSIS 1 (PRAD1)

PARATHYROID HORMONE-LIKE HORMONE (PTHLH)

PARATHYROID HORMONE-RELATED PEPTIDE (PTHrP)

P-CADHERIN

PD-ECGF

PDGF-□

PEANUT-REACTIVE URINARY MUCIN (PUM)

P-GLYCOPROTEIN (P-GP)

PGP-1

PHGS-2

PHS-2

PIP

PLAKOGLOBIN

PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 1)

PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 2)

PLASMINOGEN ACTIVATOR (TISSUE-TYPE)

PLASMINOGEN ACTIVATOR (UROKINASE-TYPE)

PLATELET GLYCOPROTEIN IIIa (GP3A)

PLAU

PLEOMORPHIC ADENOMA GENE-LIKE 1 (PLAGL1)

POLYMORPHIC EPITHELIAL MUCIN (PEM)

PRAD1

PROGESTERONE RECEPTOR (PgR)

PROGESTERONE RESISTANCE

PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-2

PROSTAGLANDIN G/H SYNTHASE-2

PROSTAGLANDIN H SYNTHASE-2

pS2

PS6K

PSORIASIN

PTHLH

PTHrP

RAD51

RAD52

RAD54

RAP46

RECEPTOR-ASSOCIATED COACTIVATOR 3 (RAC3)

REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY (REA)

S100A4

S100A6

S100A7

S6K

SART-1

SCAFFOLD ATTACHMENT FACTOR B (SAF-B)

SCATTER FACTOR (SF)

SECRETED PHOSPHOPROTEIN-1 (SPP-1)

SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE (SPARC)

STANNICALCIN

STEROID RECEPTOR COACTIVATOR-1 (SRC-1)

STEROID RECEPTOR COACTIVATOR-2 (SRC-2)

STEROID RECEPTOR COACTIVATOR-3 (SRC-3)

STEROID RECEPTOR RNA ACTIVATOR (SRA)

STROMELYSIN-1

STROMELYSIN-3

TENASCIN-C (TN-C)

TESTES-SPECIFIC PROTEASE 50

THROMBOSPONDIN I

THROMBOSPONDIN II

THYMIDINE PHOSPHORYLASE (TP)

THYROID HORMONE RECEPTOR ACTIVATOR MOLECULE 1 (TRAM-1)

TIGHT JUNCTION PROTEIN 1 (TJP1)

TIMP1

TIMP2

TIMP3

TIMP4

TISSUE-TYPE PLASMINOGEN
ACTIVATOR

TN-C

TP53

tPA

TRANSCRIPTIONAL INTERMEDIARY
FACTOR 2 (TIF2)

TREFOIL FACTOR 1 (TFF1)

TSG101

TSP-1

TSP1

TSP-2

TSP2

TSP50

TUMOR CELL COLLAGENASE
STIMULATING FACTOR (TCSF)

TUMOR-ASSOCIATED EPITHELIAL
MUCIN

uPA

uPAR

UROKINASE

UROKINASE-TYPE PLASMINOGEN
ACTIVATOR

UROKINASE-TYPE PLASMINOGEN
ACTIVATOR RECEPTOR (uPAR)

UVOMORULIN

VASCULAR ENDOTHELIAL GROWTH
FACTOR

VASCULAR ENDOTHELIAL GROWTH
FACTOR RECEPTOR-2 (VEGFR2)

VASCULAR ENDOTHELIAL GROWTH
FACTOR-A

VASCULAR PERMEABILITY FACTOR

VEGFR2

VERY LATE T-CELL ANTIGEN BETA
(VLA-BETA)

VIMENTIN

VITRONECTIN RECEPTOR ALPHA
POLYPEPTIDE (VNRA)

VITRONECTIN RECEPTOR

VON WILLEBRAND FACTOR

VPF

VWF

WNT-1

ZAC

ZO-1

ZONULA OCCLUDENS-1

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1(+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2(-)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3(+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4(-)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1(+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2(-)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3(+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4(-)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1(+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2(-)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3(+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4(-)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1(+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2(-)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3(+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4(-)	26	CATCCTCCCCTGCATGTGT	21	

Figure 11
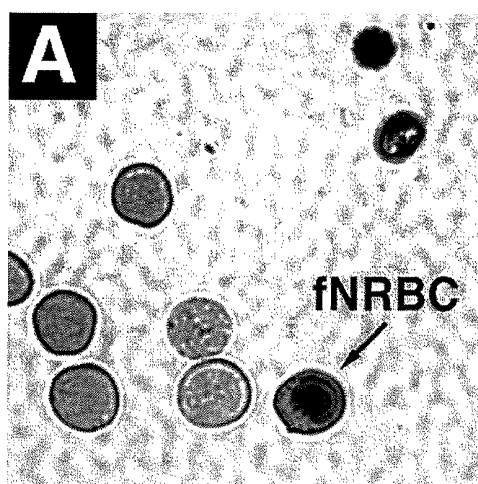
Figure 12 A
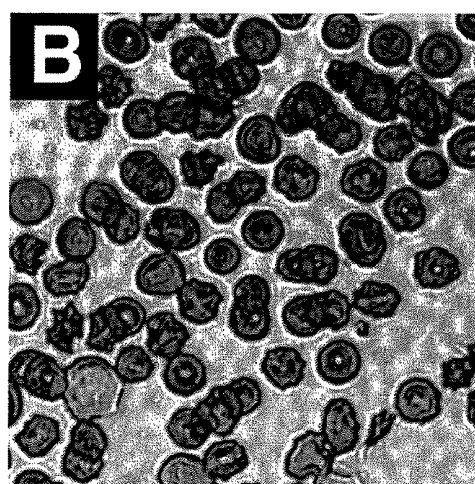
Figure 12 B
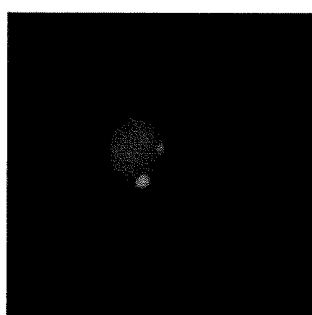
Figure 13A
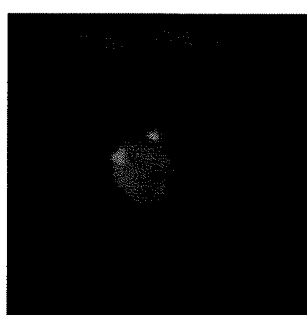
Figure 13B
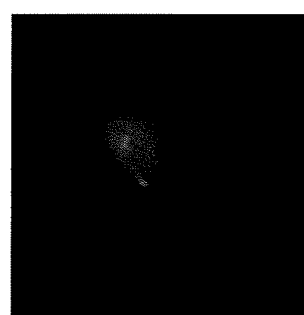
Figure 13C
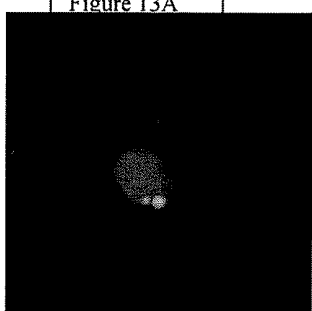
Figure 13D
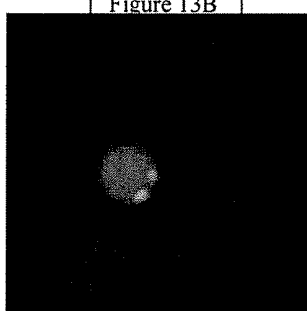
Figure 13E

Figure 13F

Figure 13A-F

Chromosome 21 Probe

X Probe

Y Probe
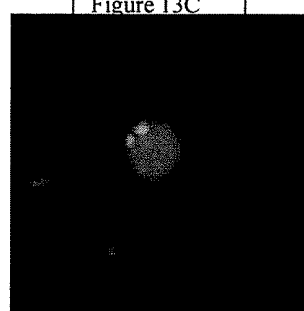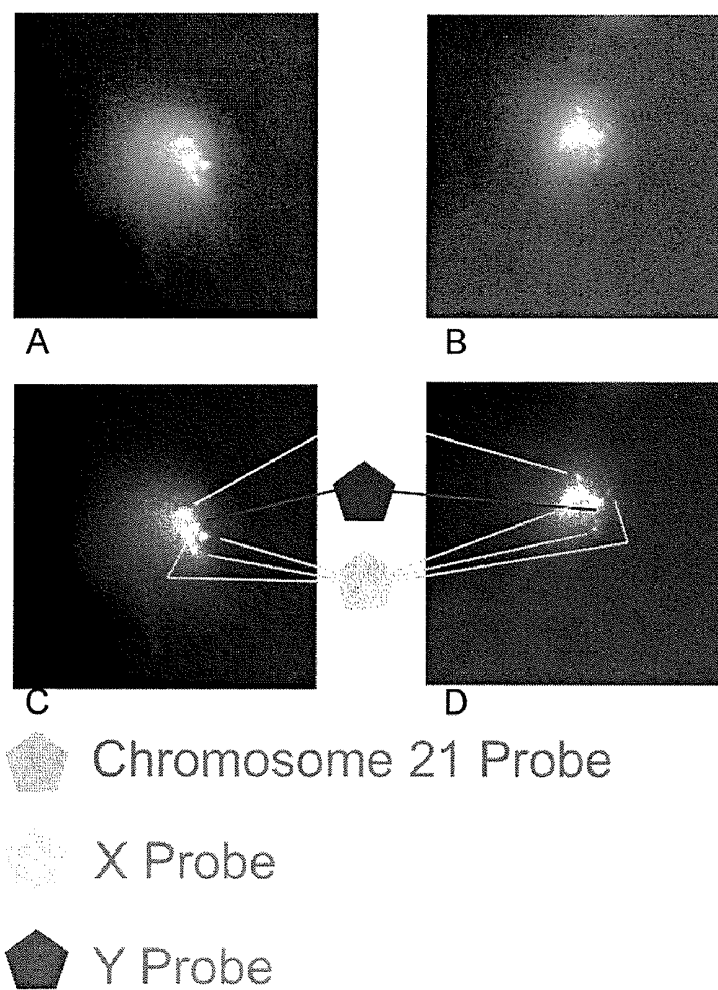
Figure 14

Figure 15

Figure 16

Product Stream   Waste Stream
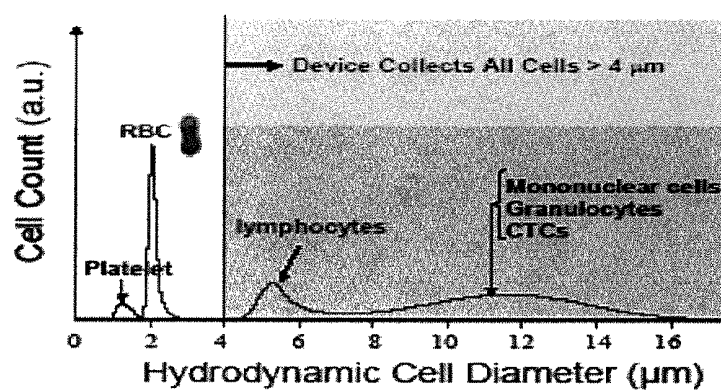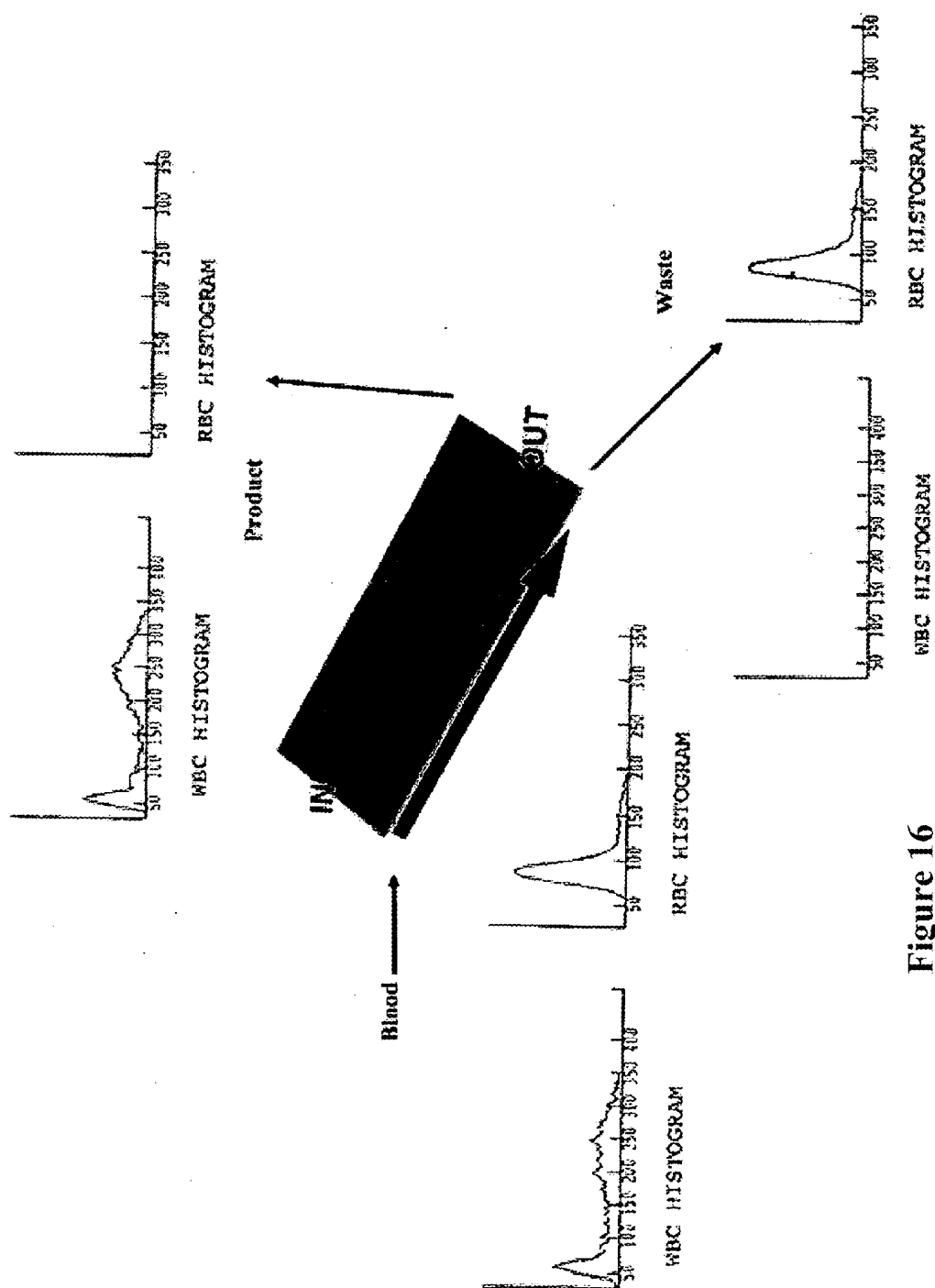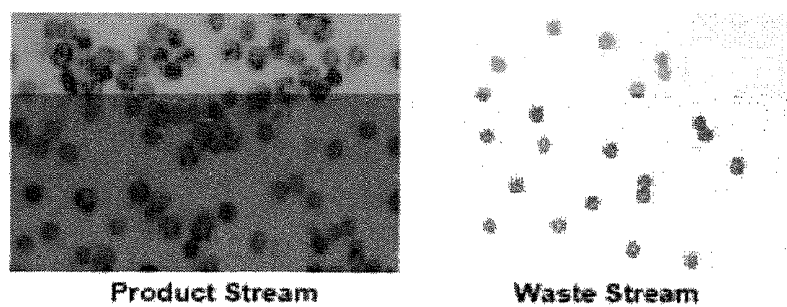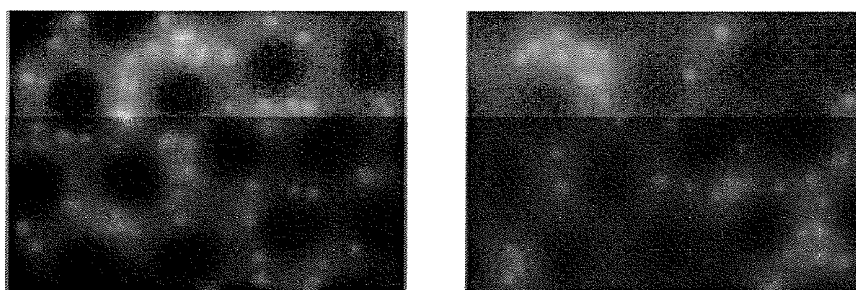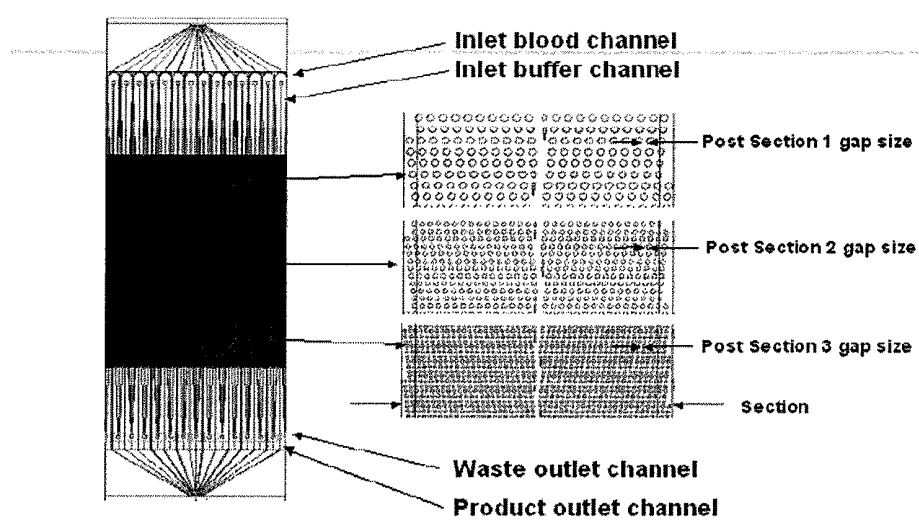
Figure 17

Figure 18

Figure 19A

	Version 1	Version 2	Version 3
Inlet channel width (blood)	50	50	100	100
Inlet channel width (buffer)	55	55	110	110
Outlet channel width (product)	49	49	98	98
Outlet channel width (waste)	50	50	100	100
Gap size / Deflect cell size				
Post section 1	18/9	36/18	44/22	50/25
Post section 2	12/6	24/12	30/15	36/18
Post section 3	8/4	16/8	20/10	24/12
Number of parallel sections	14	14	14	14
Etch depth	150	150	150	150
Product cell size (cut off)	4	8	10	12
Estimated Flow rate. ml/hr	5	10	20	30

Figure 19B

- Chip dimensions: 66.0 x 24.9 mm
- Post Field dimensions: 51.3 x 18.9 mm
- Post diameter: 104 μm
- Port dimensions
  - Front side 2.83 x2.83 mm
  - Back side 1.66 x 1.66 mm
- Substrate: Silicon
- Etch depth: 100 μm
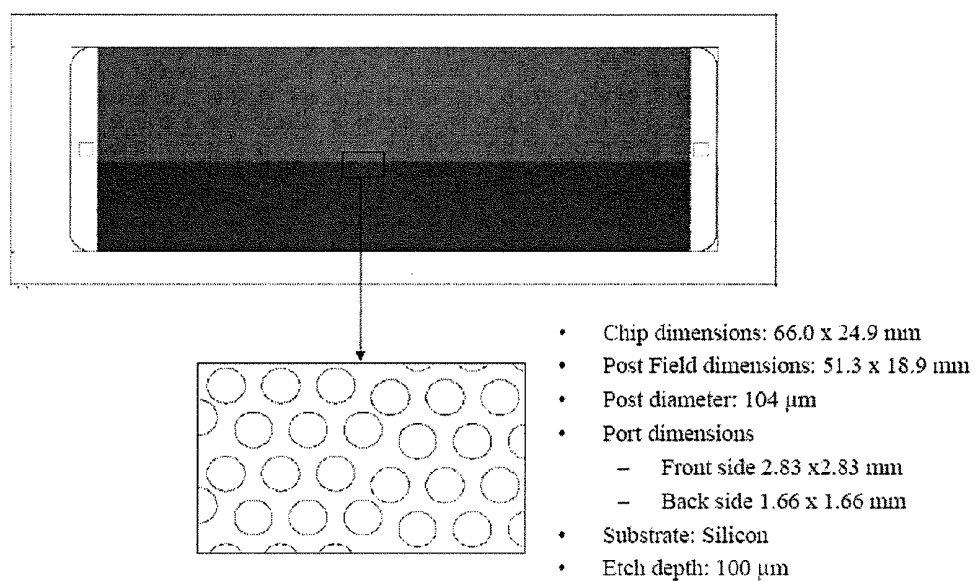
Figure 19C
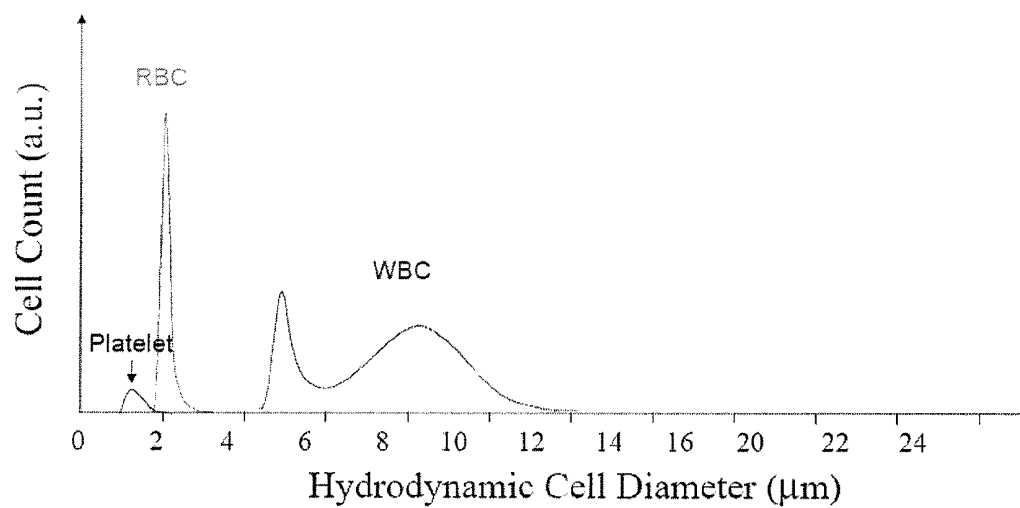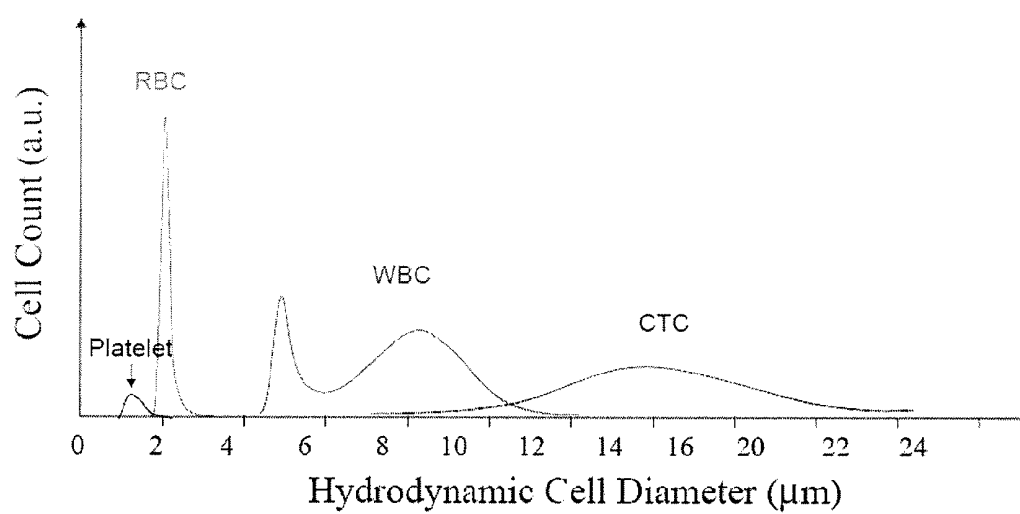
Figure 20A

Figure 20B

Figure 20C

Figure 20D

Whole Blood

Waste

Large cells

Molecular Analysis
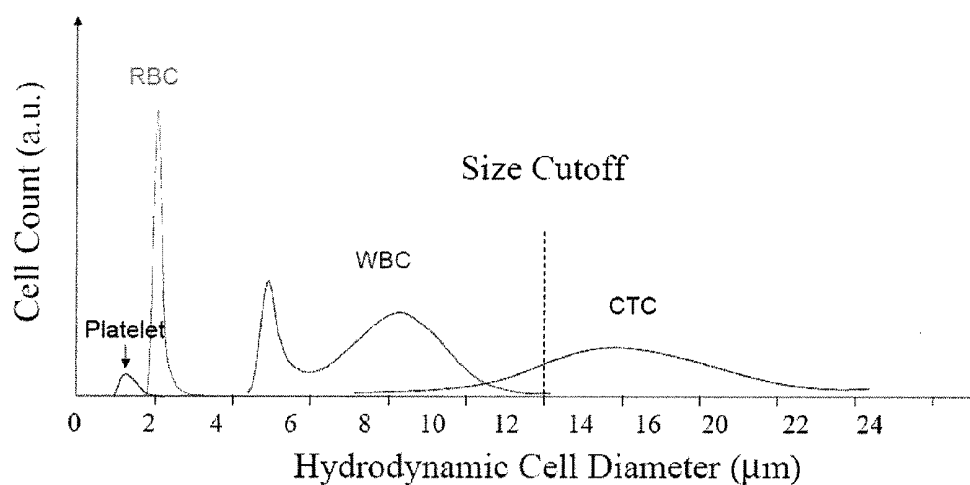
Figure 21

Figure 22

Figure 23

Figure 24.

CAA → CAG (silent) at 2361   CTG → CGG (L828R) at 2573   ACC → ACT (silent) at 2709
ACG → ATG (T790M) at 2369

Figure 25

WBC cDNA	−	+	−	−	+	−	−	+	−	−	+	−
H1650 cDNA	−	−	+	−	−	+	−	−	+	−	−	+
Target mRNA	BCKD			CD45			EpCaM			EGFR		

Figure 26A

	Tissue	Ct	[mRNA]
1	Renal Blood Vessel	24.02	25,916
2	Blood Mononuclear Cells (PBMC)	>40	0
3	Colon	24.28	21,968
4	Primary Lung Bronchus	24.49	19,224

Figure 26B

PCR #1   PCR #2 (Nested)
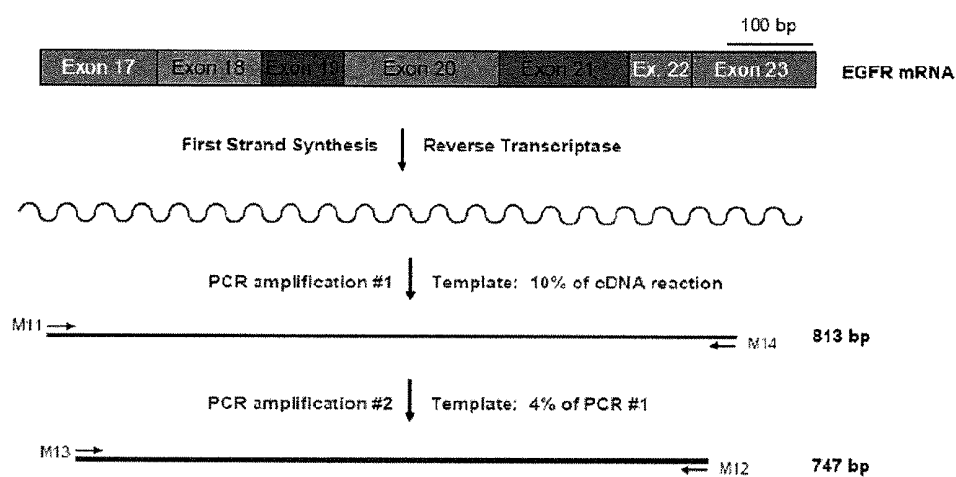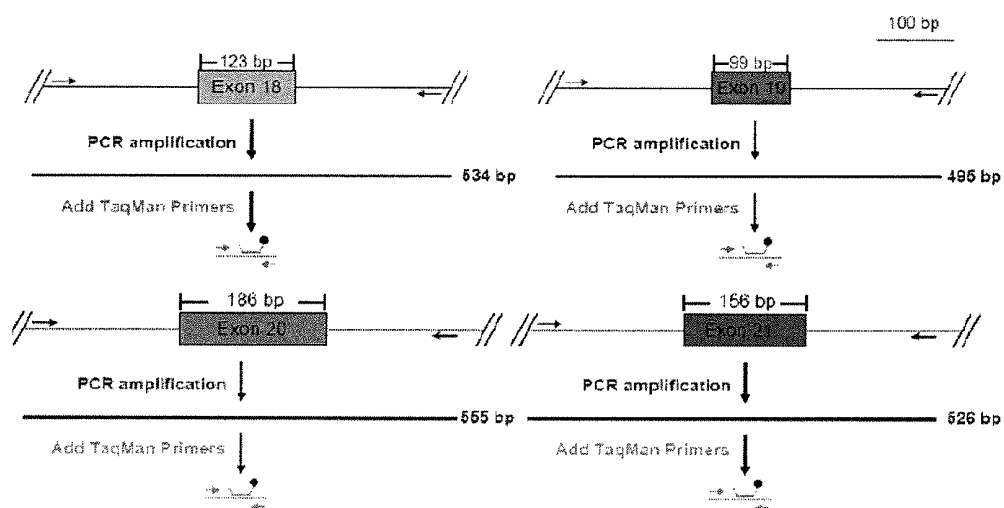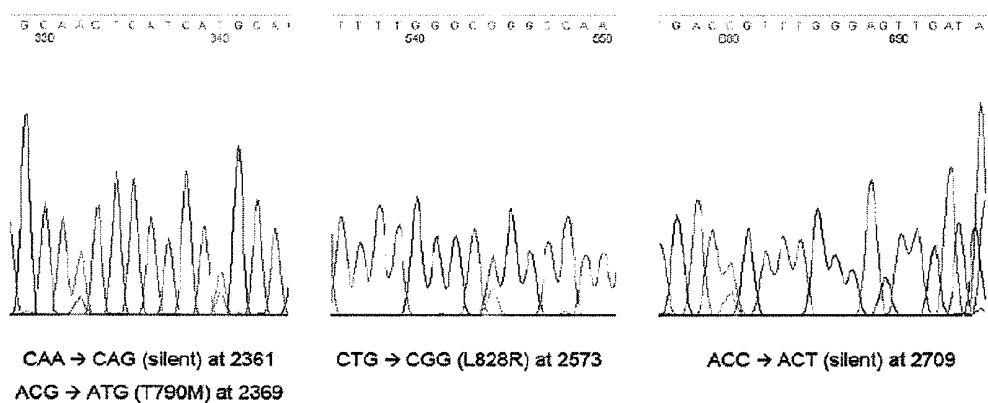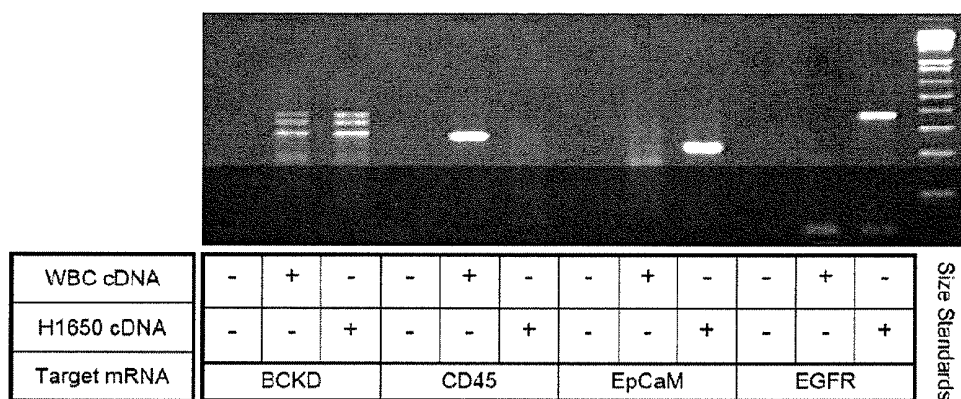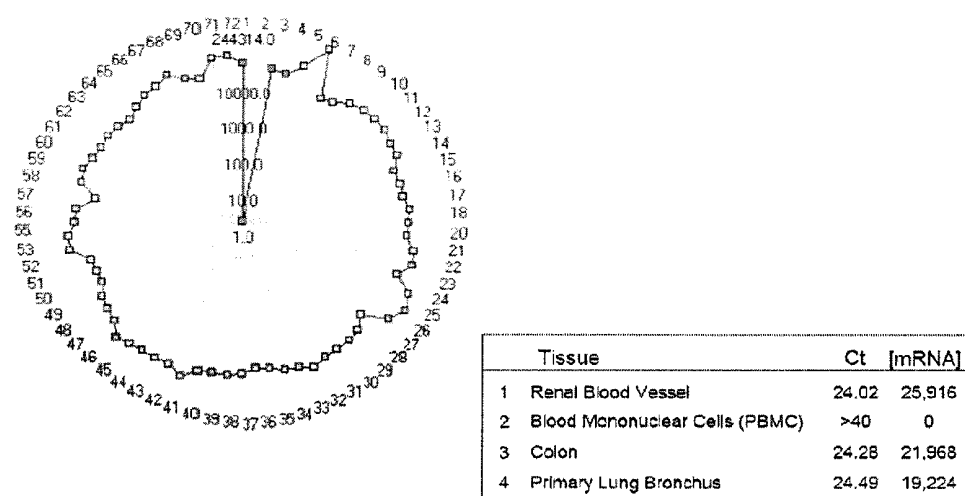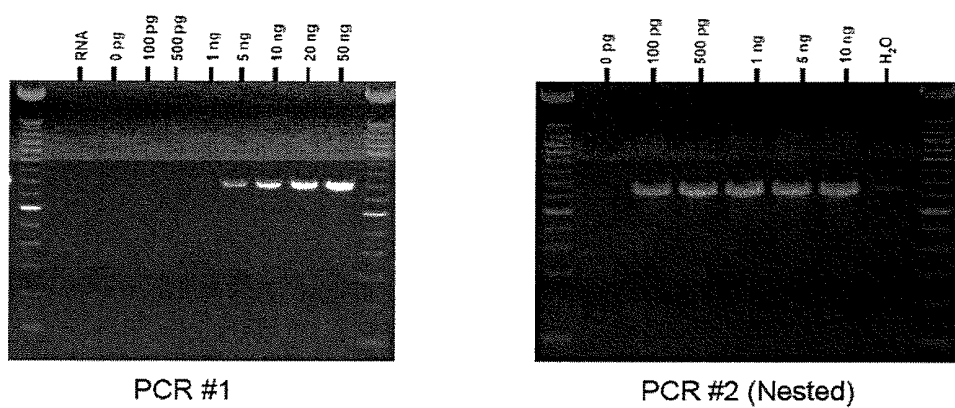
NIH-3T3	--	100 pg total RNA = ~20 cells
NCI-H1975	--	100 pg total RNA = ~10 cells
COS-7	--	100 pg total RNA = ~6 cells

Figure 27

1000 ng PBMC RNA (no cDNA Rx)

1 ng H1975 plus

PBMC RNA

cDNA Template:

Mock cDNA
(No RNA)

1 ng   10 ng   100 ng   1000 ng

1 ng H1975 alone

200 ng H1975 RNA (no cDNA Rx)

H₂O Control

Spurious PBMC
amplicons
visible at high
dilution

813 bp

813 bp

Figure 28A

Mock cDNA (No RNA)

1000 ng PBMC RNA

1 ng H1975 plus:

1 ng PBMC

10 ng PBMC

100 ng PBMC

1000 ng PBMC

1 ng H1975 alone

200 ng H1975 RNA

H₂O Control of 1° PCR

H₂O Control

Template: 2 µl
(4%) of the
corresponding 1°
PCR Reaction

Specific amplicon
produced even at
1000:1 dilution

747 bp

Figure 28B

Figure 29

Figure 30

Figure 31

FIGURE 32

FIGURE 33

FIGURE 34

FIGURE 35

RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS

CROSS-REFERENCE

[0001] This application is a continuation application of Ser. No. 11/763,421, filed Jun. 14, 2007, which is incorporated herein by reference in its entirety and to which application we claim priority under 35 USC §120.

[0002] This application claims the benefit of U.S. Provisional Application No. 60/804,819, filed Jun. 14, 2006, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0003] Analysis of specific cells can give insight into a variety of diseases. These analyses can provide non-invasive tests for detection, diagnosis and prognosis of diseases such as cancer or fetal disorders, thereby eliminating the risk of invasive diagnosis. Regarding fetal disorders, current prenatal diagnosis, such as amniocentesis and chorionic villus sampling (CVS), are potentially harmful to the mother and to the fetus. The rate of miscarriage for pregnant women undergoing amniocentesis is increased by 0.5-1%, and that figure is slightly higher for CVS. Because of the inherent risks posed by amniocentesis and CVS, these procedures are offered primarily to older women, e.g., those over 35 years of age, who have a statistically greater probability of bearing children with congenital defects. As a result, a pregnant woman at the age of 35 has to balance an average risk of 0.5-1% to induce an abortion by amniocentesis against an age related probability for trisomy 21 of less than 0.3%.
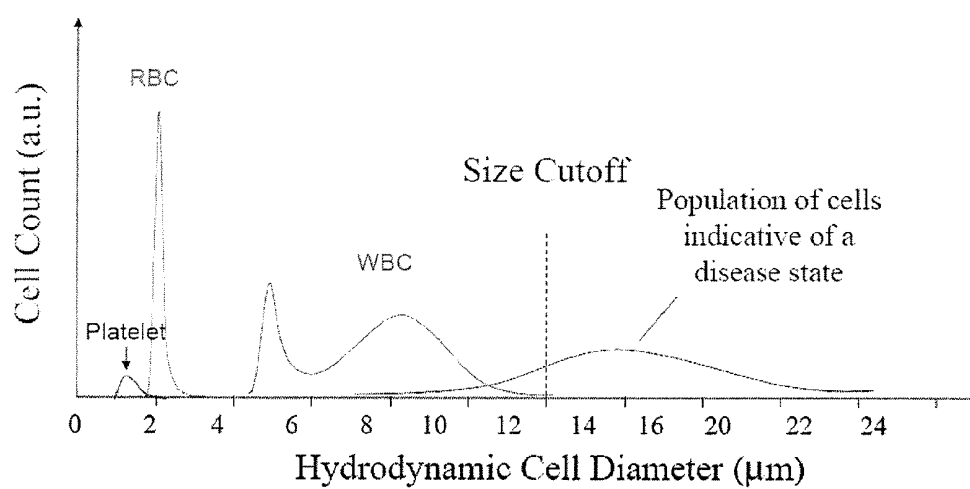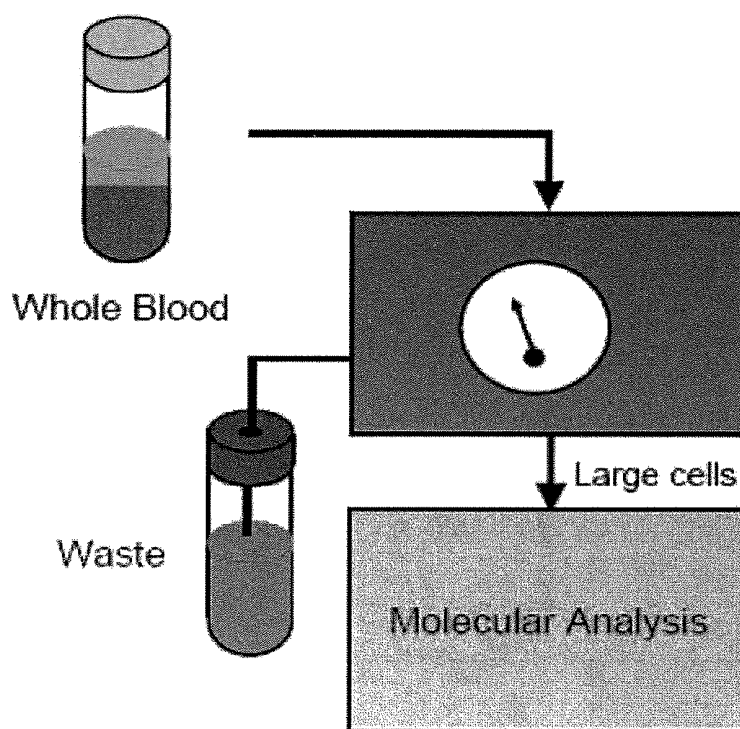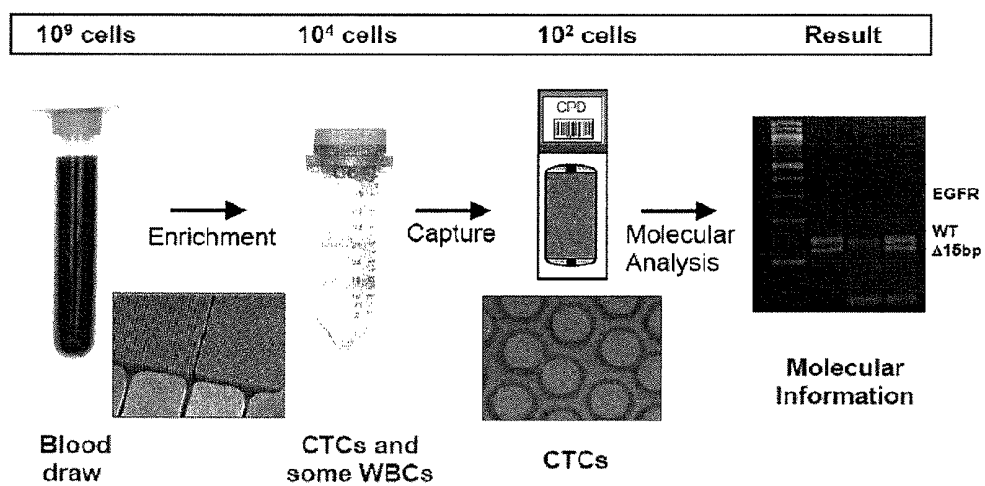
[0004] Regarding prenatal diagnostics, some non-invasive methods have already been developed to screen for fetuses at higher risk of having specific congenital defects. For example, maternal serum alpha-fetoprotein, and levels of unconjugated estriol and human chorionic gonadotropin can be used to identify a proportion of fetuses with Down's syndrome. However, these tests suffer from many false positive. Similarly, ultrasonography is used to determine congenital defects involving neural tube defects and limb abnormalities, but such methods are limited to time periods after fifteen weeks of gestation and are present unreliable results.

[0005] The presence of fetal cells within the blood of pregnant women offers the opportunity to develop a prenatal diagnostic that replaces amniocentesis and thereby eliminates the risk of today's invasive diagnosis. However, fetal cells represent a small number of cells against the background of a large number of maternal cells in the blood which make the analysis time consuming and prone to error.

[0006] With respect to cancer diagnosis, early detection is of paramount importance. Cancer is a disease marked by the uncontrolled proliferation of abnormal cells. In normal tissue, cells divide and organize within the tissue in response to signals from surrounding cells. Cancer cells do not respond in the same way to these signals, causing them to proliferate and, in many organs, form a tumor. As the growth of a tumor continues, genetic alterations may accumulate, manifesting as a more aggressive growth phenotype of the cancer cells. If left untreated, metastasis, the spread of cancer cells to distant areas of the body by way of the lymph system or bloodstream, may ensue. Metastasis results in the formation of secondary tumors at multiple sites, damaging healthy tissue. Most cancer death is caused by such secondary tumors. Despite decades of advances in cancer diagnosis and therapy, many cancers continue to go undetected until late in their development. As one example, most early-stage lung cancers are asymptomatic and are not detected in time for curative treatment, resulting in an overall five-year survival rate for patients with lung cancer of less than 15%. However, in those instances in which lung cancer is detected and treated at an early stage, the prognosis is much more favorable.

[0007] The methods of the present invention allow for the detection of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In addition, the methods of the present invention can also be utilized to detect or diagnose cancer.

SUMMARY OF THE INVENTION

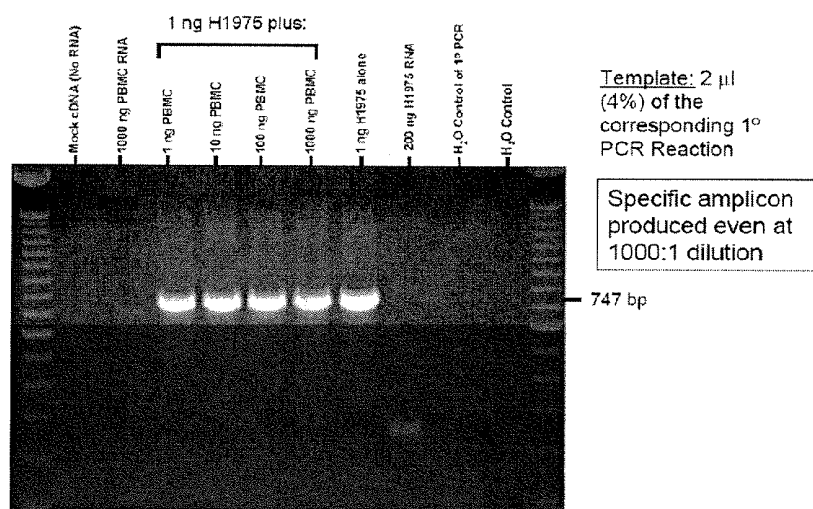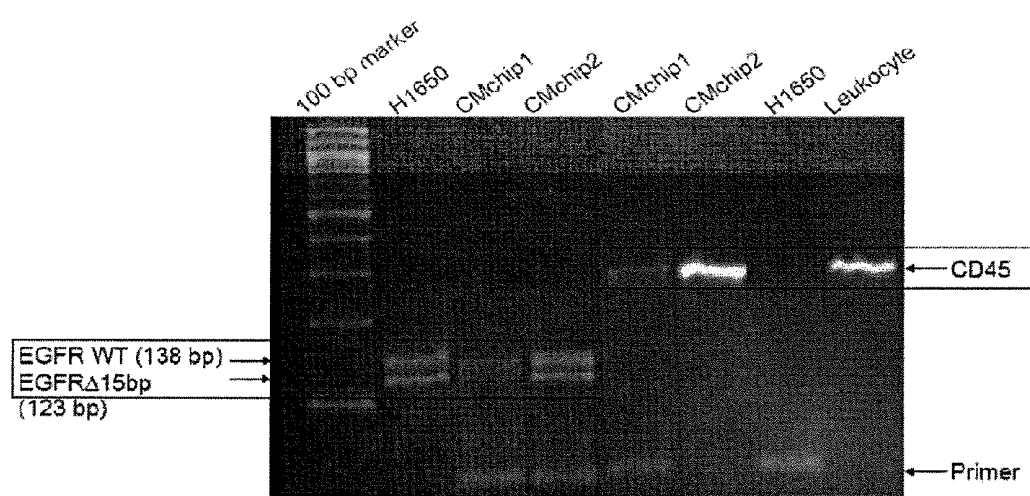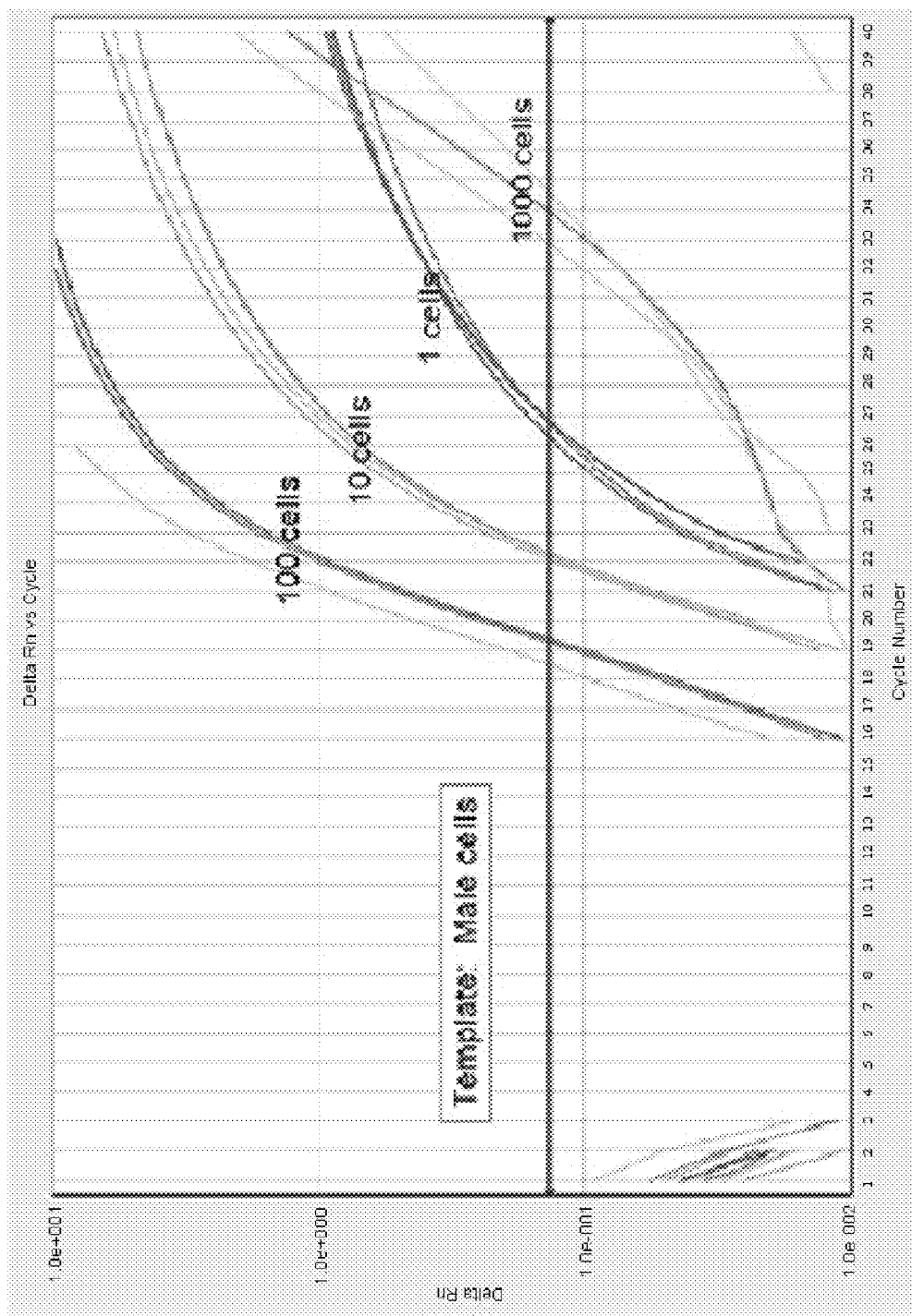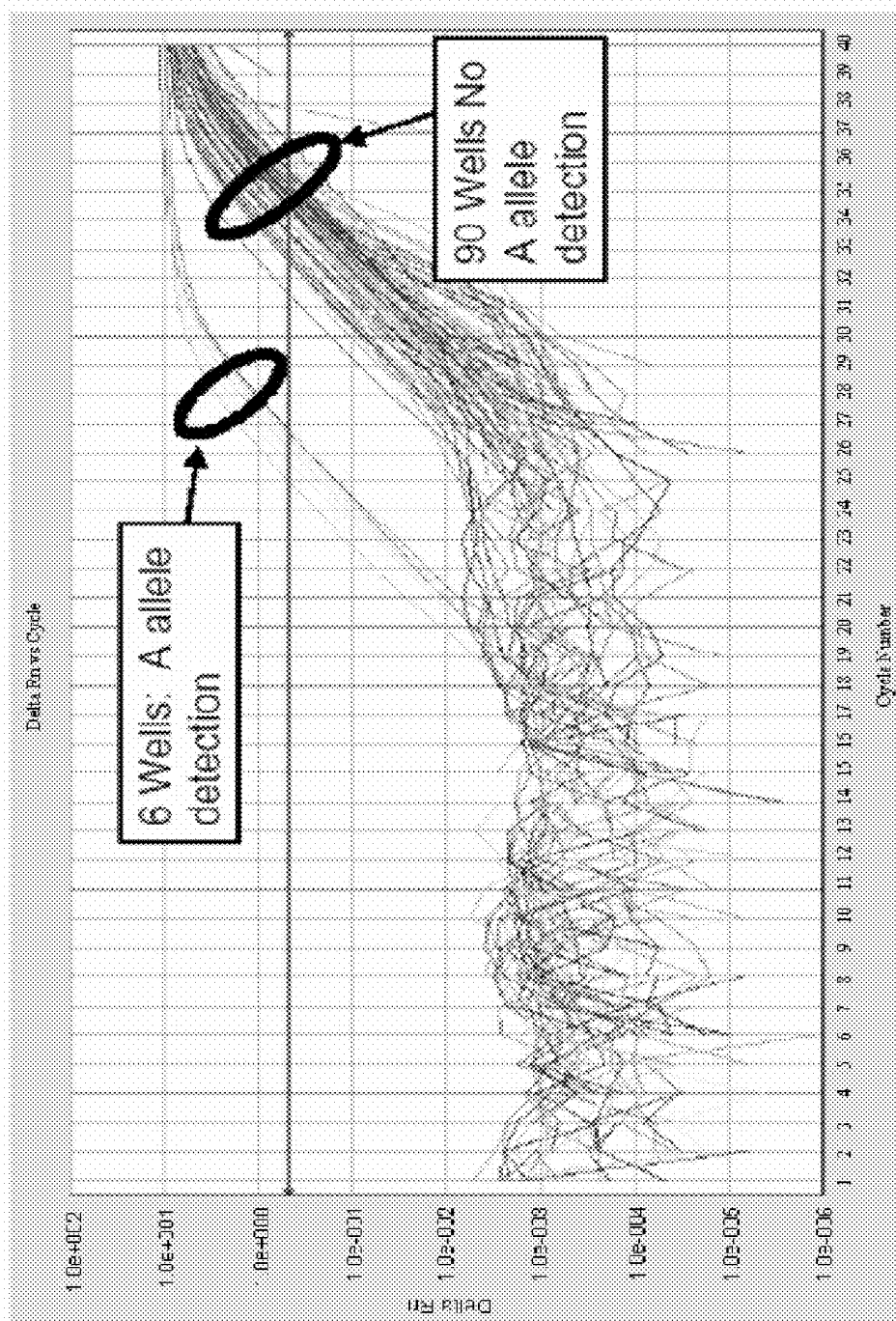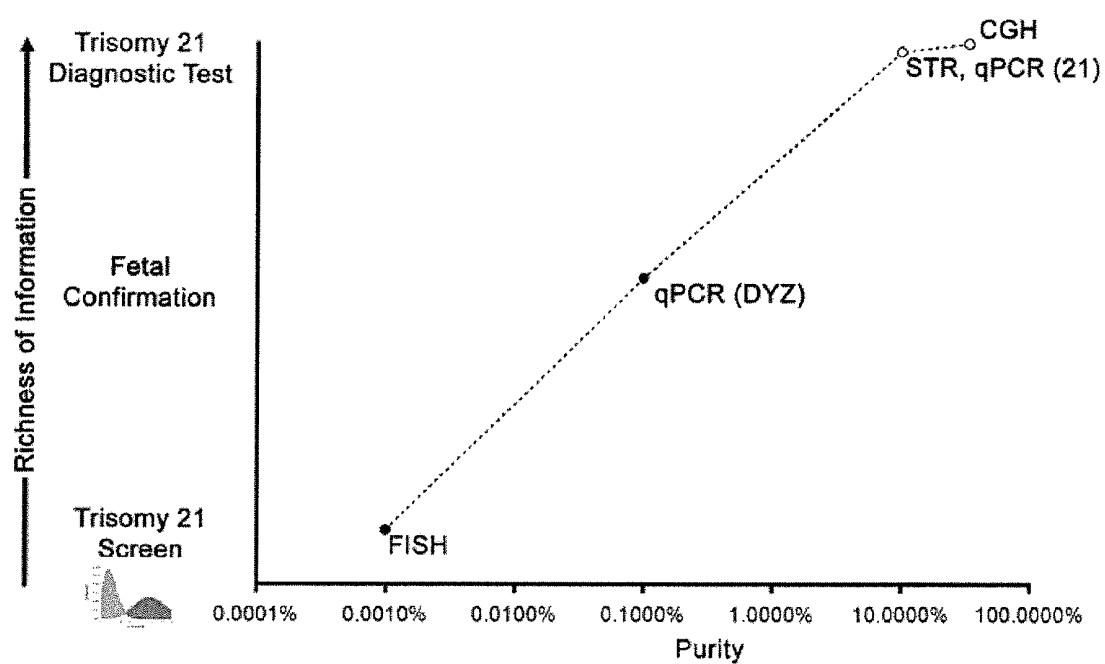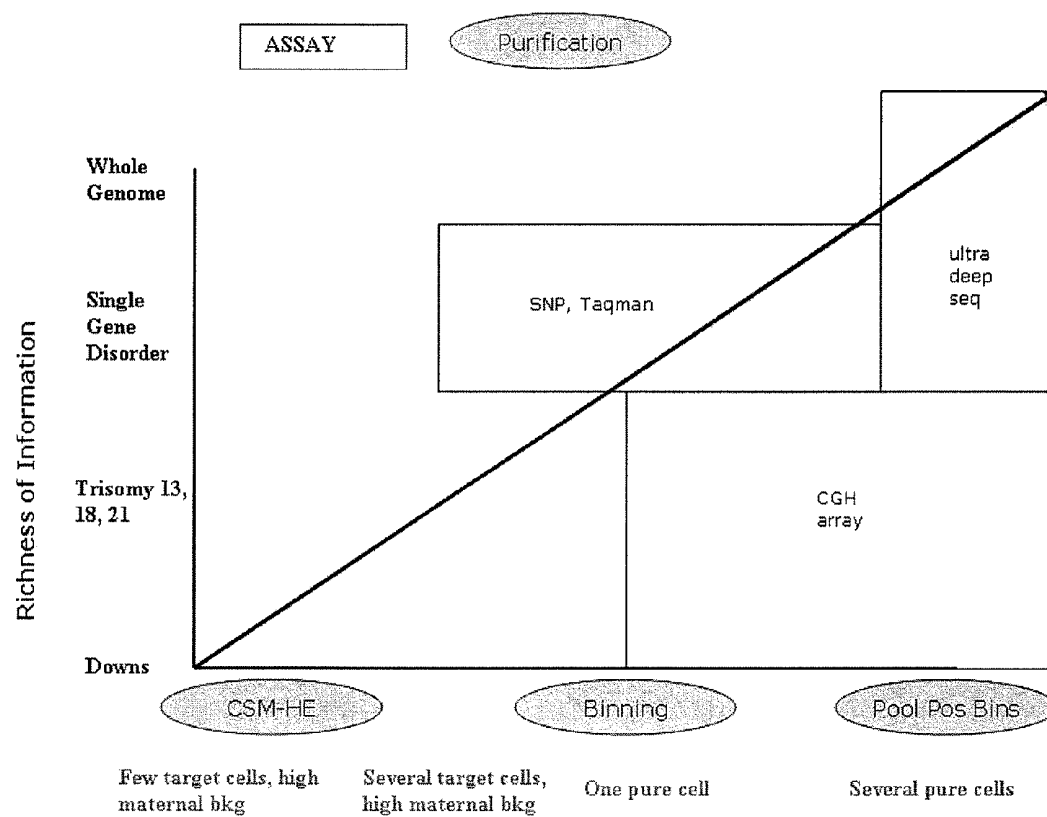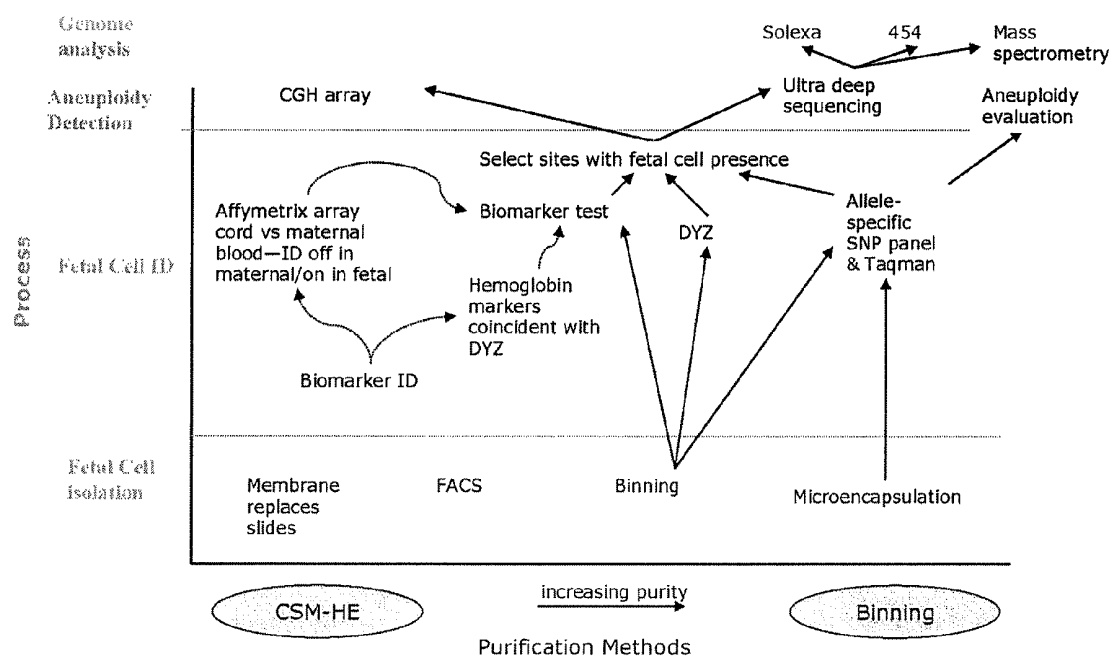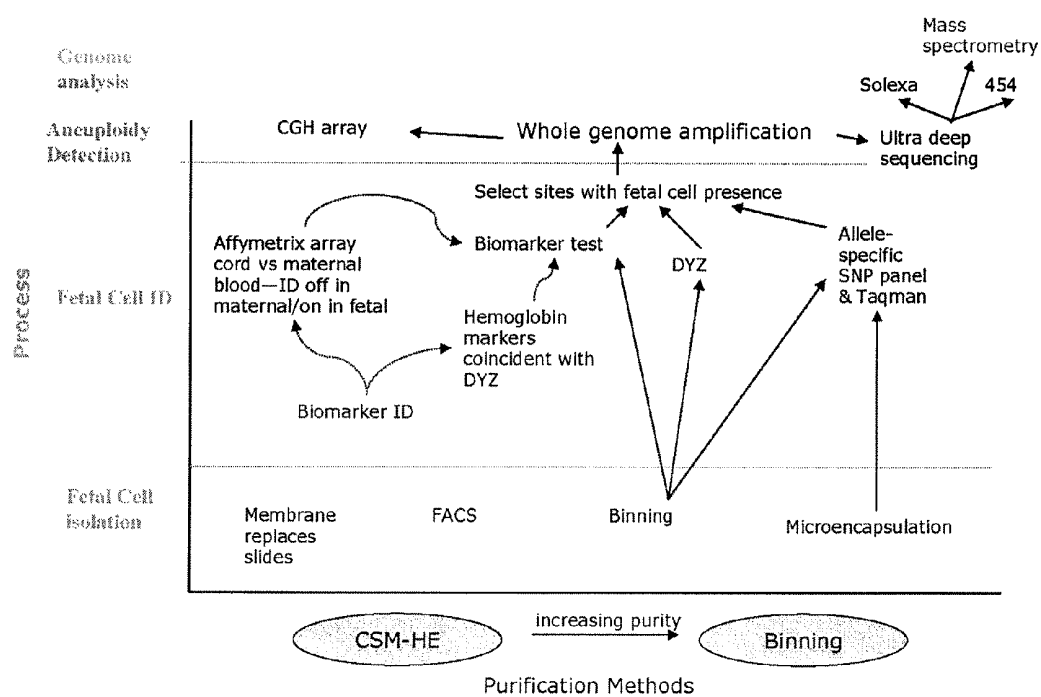[0008] The present invention relates to methods for the detection of fetal cells or cancer cells in a mixed sample. In one embodiment, the present invention provides methods for determining fetal abnormalities in a sample comprising fetal cells that are mixed with a population of maternal cells. In some embodiments, determining the presence of fetal cells and fetal abnormalities comprises labeling one or more regions of genomic DNA in each cell from a mixed sample comprising at least one fetal cell with different labels wherein each label is specific to each cell. In some embodiments, the genomic DNA to be labeled comprises one or more polymorphisms, particularly STRs or SNPs

[0009] In some embodiments, the methods of the invention allow for simultaneously detecting the presence of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In some embodiments, the sample is enriched to contain at least one fetal and one non fetal cell, and in other embodiments, the cells of the enriched population can be divided between two or more discrete locations that can be used as addressable locations. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0010] In some embodiments, the methods comprise labeling one or more regions of genomic DNA in each cell in the enriched sample with different labels, wherein each label is specific to each cell, and quantifying the labeled DNA regions. The labeling methods can comprise adding a unique tag sequence for each cell in the mixed sample. In some embodiments, the unique tag sequence identifies the presence or absence of a DNA polymorphism in each cell from the mixed sample. Labels are added to the cells/DNA using an amplification reaction, which can be performed by PCR methods. For example, amplification can be achieved by multiplex PCR. In some embodiments, a further PCR amplification is performed using nested primers for the genomic DNA region(s).

[0011] In some embodiments, the DNA regions can be amplified prior to being quantified. The labeled DNA can be quantified using sequencing methods, which, in some embodiments, can precede amplifying the DNA regions. The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be used to declare the presence of fetal cells and to determine the copy numbers of

the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0012] The invention also relates to methods of identifying cells from a mixed sample with non-maternal genomic DNA and identifying said cells with non-maternal genomic DNA as fetal cells. In some embodiments, the ratio of maternal to paternal alleles is compared on the identified fetal cells in the mixed sample.

[0013] In one embodiment, the invention provides for a method for determining a fetal abnormality in a maternal sample that comprises at least one fetal and one non fetal cell. The sample can be enriched to contain at least one fetal cell, and the enriched maternal sample can be arrayed into a plurality of discrete sites. In some embodiments, each discrete site comprises no more than one cell.

[0014] In some embodiments, the invention comprises labeling one or more regions of genomic DNA from the arrayed samples using primers that are specific to each DNA region or location, amplifying the DNA region(s), and quantifying the labeled DNA region. The labeling of the DNA region(s) can comprise labeling each region with a unique tag sequence, which can be used to identify the presence or absence of a DNA polymorphism on arrayed cells and the distinct location of the cells.

[0015] The step of determining can comprise identifying non-maternal alleles at the distinct locations, which can result from comparing the ratio of maternal to paternal alleles at the location. In some embodiments, the method of identifying a fetal abnormality in an arrayed sample can further comprise amplifying the genomic DNA regions. The genomic DNA regions can comprise one or more polymorphisms e.g. STRs and SNPs, which can be amplified using PCR methods including multiplex PCR. An additional amplification step can be performed using nested primers.

[0016] The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be sued to declare the presence of fetal cells and to determine the copy numbers of the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0017] In one embodiment, the invention provides methods for diagnosing a cancer and giving a prognosis by obtaining and enriching a blood sample from a patient for epithelial cells, splitting the enriched sample into discrete locations, and performing one or more molecular and/or morphological analyses on the enriched and split sample. The molecular analyses can include detecting the level of expression or a mutation of gene disclosed in FIG. 10. Preferably, the method comprises performing molecular analyses on EGFR, EpCAM, GA733-2, MUC-1, HER-2, or Claudin-7 in each arrayed cell. The morphological analyses can include identifying, quantifying and/or characterizing mitochondrial DNA, telomerase, or nuclear matrix proteins.

[0018] In some embodiments, the sample can be enriched for epithelial cells by at least 10,000 fold, and the diagnosis and prognosis can be provided prior to treating the patient for the cancer. Preferably, the blood samples are obtained from a patient at regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[0019] In some embodiments, the step of enriching a patient's blood sample for epithelial cells involves flowing the sample through a first array of obstacles that selectively directs cells that are larger than a predetermined size to a first outlet and cells that are smaller than a predetermined size to a second outlet. Optionally, the sample can be subjected to further enrichment by flowing the sample through a second array of obstacles, which can be coated with antibodies that selectively bind to white blood cells or epithelial cells. For example, the obstacles of the second array can be coated with anti-EpCAM antibodies.

[0020] Splitting the sample of cells of the enriched population can comprises splitting the enriched sample to locate individual cells at discrete sites that can be addressable sites. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0021] In some embodiments there are provided kits comprising devices for enriching the sample and the devices and reagents needed to perform the genetic analysis. The kits may contain the arrays for size-based separation, reagents for uniquely labeling the cells, devices for splitting the cells into individual addressable locations and reagents for the genetic analysis.

SUMMARY OF THE DRAWINGS

[0022] FIGS. 1A-1D illustrate various embodiments of a size-based separation module.

[0023] FIGS. 2A-2C illustrate one embodiment of an affinity separation module.

[0024] FIG. 3 illustrate one embodiment of a magnetic separation module.

[0025] FIG. 4 illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

[0026] FIG. 5 illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

[0027] FIG. 6 illustrates an overview for diagnosing, prognosing or monitoring cancer in a patient.

[0028] FIGS. 7A-7B illustrate an assay using molecular inversion probes (SEQ ID NO: 67). FIG. 7C illustrates an overview of the use of nucleic acid tags.

[0029] FIGS. 8A-8C illustrate one example of a sample splitting apparatus.

[0030] FIG. 9 illustrates the probability of having 2 or more CTC's loaded into a single sample well.

[0031] FIG. 10 illustrates genes whose expression or mutations can be associated with cancer or another condition diagnosed herein.

[0032] FIG. 11 illustrates primers useful in the methods herein.

[0033] FIGS. 12A-B illustrate cell smears of the product and waste fractions.

[0034] FIGS. 13A-F illustrate isolated fetal cells confirmed by the reliable presence of male cells.

[0035] FIG. 14 illustrates cells with abnormal trisomy 21 pathology.

[0036] FIG. 15 illustrates performance of a size-based separation module.

[0037] FIG. 16 illustrates histograms of these cell fractions resulting from a size-based separation module.

[0038] FIG. 17 illustrates a first output and a second output of a size-based separation module.

[0039] FIG. 18 illustrates epithelial cells bound to a capture module of an array of obstacles coated with anti-EpCAM.

3

[0040] FIGS. 19A-C illustrate one embodiment of a flow-through size-based separation module adapted to separate epithelial cells from blood and alternative parameters that can be used with such device.

[0041] FIGS. 20A-D illustrate various targeted subpopulations of cells that can be isolated using size-based separation and various cut-off sizes that can be used to separate such targeted subpopulations.

[0042] FIG. 21 illustrates a device of the invention with counting means to determine the number of cells in the enriched sample.

[0043] FIG. 22 illustrates an overview of one aspect of the invention for diagnosing, prognosing, or monitoring cancer in a patient.

[0044] FIG. 23 illustrates the use of EGFR mRNA for generating sequencing templates.

[0045] FIG. 24 illustrates performing real-time quantitative allele-specific PCR reactions to confirm the sequence of mutations in EGFR mRNA.

[0046] FIG. 25 illustrates confirmation of the presence of a mutation is when the signal from a mutant allele probe rises above the background level of fluorescence.

[0047] FIG. 25 illustrates confirmation of the presence of a mutation is when the signal from a mutant allele probe rises above the background level of fluorescence (SEQ ID NOS 68-70, respectively in order of appearance from left to right).

[0048] FIG. 27 illustrate results of the first and second EGFR PCR reactions.

[0049] FIGS. 28A-B results of the first and second EGFR PCR reactions.

[0050] FIG. 29 illustrates that EGFR wild type and mutant amplified fragments are readily detected, despite the high leukocyte background.

[0051] FIG. 30 illustrates the detection of single copies of a fetal cell genome by qPCR.

[0052] FIG. 31 illustrates detection of single fetal cells in binned samples by SNP analysis.

[0053] FIG. 32 illustrates a method of trisomy testing. The trisomy 21 screen is based on scoring of target cells obtained from maternal blood. Blood is processed using a cell separation module for hemoglobin enrichment (CSM-HE). Enriched cells are transferred to slides that are first stained and subsequently probed by FISH. Images are acquired, such as from bright field or fluorescent microscopy, and scored. The proportion of trisomic cells of certain classes serves as a classifier for risk of fetal trisomy 21. Fetal genome identification can performed using assays such as: (1) STR markers; (2) qPCR using primers and probes directed to loci, such as the multi-repeat DYZ locus on the Y-chromosome; (3) SNP detection; and (4) CGH (comparative genome hybridization) array detection.

[0054] FIG. 33 illustrates assays that can produce information on the presence of aneuploidy and other genetic disorders in target cells. Information on aneuploidy and other genetic disorders in target cells may be acquired using technologies such as: (1) a CGH array established for chromosome counting, which can be used for aneuploidy determination and/or detection of intra-chromosomal deletions; (2) SNP/taqman assays, which can be used for detection of single nucleotide polymorphisms; and (3) ultra-deep sequencing, which can be used to produce partial or complete genome sequences for analysis.

[0055] FIG. 34 illustrates methods of fetal diagnostic assays. Fetal cells are isolated by CSM-HE enrichment of target cells from blood. The designation of the fetal cells may be confirmed using techniques comprising FISH staining (using slides or membranes and optionally an automated detector), FACS, and/or binning. Binning may comprise distribution of enriched cells across wells in a plate (such as a 96 or 384 well plate), microencapsulation of cells in droplets that are separated in an emulsion, or by introduction of cells into microarrays of nanofluidic bins. Fetal cells are then identified using methods that may comprise the use of biomarkers (such as fetal (gamma) hemoglobin), allele-specific SNP panels that could detect fetal genome DNA, detection of differentially expressed maternal and fetal transcripts (such as Affymetrix chips), or primers and probes directed to fetal specific loci (such as the multi-repeat DYZ locus on the Y-chromosome). Binning sites that contain fetal cells are then be analyzed for aneuploidy and/or other genetic defects using a technique such as CGH array detection, ultra deep sequencing (such as Solexa, 454, or mass spectrometry), STR analysis, or SNP detection.

[0056] FIG. 35 illustrates methods of fetal diagnostic assays, further comprising the step of whole genome amplification prior to analysis of aneuploidy and/or other genetic defects.

INCORPORATION BY REFERENCE

[0057] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

DETAILED DESCRIPTION OF THE INVENTION

[0058] The present invention provides systems, apparatus, and methods to detect the presence of or abnormalities of rare analytes or cells, such as hematapoeitic bone marrow progenitor cells, endothelial cells, fetal cells, epithelial cells, or circulating tumor cells in a sample of a mixed analyte or cell population (e.g., maternal peripheral blood samples).

[0059] I. Sample Collection/Preparation

[0060] Samples containing rare cells can be obtained from any animal in need of a diagnosis or prognosis or from an animal pregnant with a fetus in need of a diagnosis or prognosis. In one example, a sample can be obtained from animal suspected of being pregnant, pregnant, or that has been pregnant to detect the presence of a fetus or fetal abnormality. In another example, a sample is obtained from an animal suspected of having, having, or an animal that had a disease or condition (e.g. cancer). Such condition can be diagnosed, prognosed, monitored and therapy can be determined based on the methods and systems herein. Animal of the present invention can be a human or a domesticated animals such as a cow, chicken, pig, horse, rabbit, dogs, cat, or goat. Samples derived from an animal or human can include, e.g., whole blood, sweat, tears, ear flow, sputum, lymph, bone marrow suspension, lymph, urine, saliva, semen, vaginal flow, cerebrospinal fluid, brain fluid, ascites, milk, secretions of the respiratory, intestinal or genitourinary tracts fluid.

[0061] To obtain a blood sample, any technique known in the art may be used, e.g. a syringe or other vacuum suction device. A blood sample can be optionally pre-treated or processed prior to enrichment. Examples of pre-treatment steps include the addition of a reagent such as a stabilizer, a preservative, a fixant, a lysing reagent, a diluent, an anti-apop-

4

totic reagent, an anti-coagulation reagent, an anti-thrombotic reagent, magnetic property regulating reagent, a buffering reagent, an osmolality regulating reagent, a pH regulating reagent, and/or a cross-linking reagent.

[0062] When a blood sample is obtained, a preservative such an anti-coagulation agent and/or a stabilizer is often added to the sample prior to enrichment. This allows for extended time for analysis/detection. Thus, a sample, such as a blood sample, can be enriched and/or analyzed under any of the methods and systems herein within 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 12 hrs, 6 hrs, 3 hrs, 2 hrs, or 1 hr from the time the sample is obtained.

[0063] In some embodiments, a blood sample can be combined with an agent that selectively lyses one or more cells or components in a blood sample. For example, fetal cells can be selectively lysed releasing their nuclei when a blood sample including fetal cells is combined with deionized water. Such selective lysis allows for the subsequent enrichment of fetal nuclei using, e.g., size or affinity based separation. In another example platelets and/or enucleated red blood cells are selectively lysed to generate a sample enriched in nucleated cells, such as fetal nucleated red blood cells (fnRBC's), maternal nucleated blood cells (mnBC), epithelial cells and circulating tumor cells. fnRBC's can be subsequently separated from mnBC's using, e.g., antigen-i affinity or differences in hemoglobin

[0064] When obtaining a sample from an animal (e.g., blood sample), the amount can vary depending upon animal size, its gestation period, and the condition being screened. In some embodiments, up to 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mL of a sample is obtained. In some embodiments, 1-50, 2-40, 3-30, or 4-20 mL of sample is obtained. In some embodiments, more than 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mL of a sample is obtained.

[0065] To detect fetal abnormality, a blood sample can be obtained from a pregnant animal or human within 36, 24, 22, 20, 18, 16, 14, 12, 10, 8, 6 or 4 weeks of gestation.

[0066] II. Enrichment

[0067] A sample (e.g. blood sample) can be enriched for rare analytes or rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells) using one or more any methods known in the art (e.g. Guetta, E M et al. Stem Cells Dev, 13(1):93-9 (2004)) or described herein. The enrichment increases the concentration of rare cells or ratio of rare cells to non-rare cells in the sample. For example, enrichment can increase concentration of an analyte of interest such as a fetal cell or epithelial cell or CTC by a factor of at least 2, 4, 6, 8, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 2,000,000, 5,000,000, 10,000,000, 20,000,000, 50,000,000, 100,000,000, 200,000,000, 500,000,000, 1,000,000,000, 2,000,000,000, or 5,000,000,000 fold over its concentration in the original sample. In particular, when enriching fetal cells from a maternal peripheral venous blood sample, the initial concentration of the fetal cells may be about 1:50,000,000 and it may be increased to at least 1:5,000 or 1:500. Enrichment can also increase concentration of rare cells in volume of rare cells/total volume of sample (removal of fluid). A fluid sample (e.g., a blood sample) of greater than 10, 15, 20, 50, or 100 mL total volume comprising rare components of interest, and it can be concentrated such that the rare component of interest into a concentrated solution of less than 0.5, 1, 2, 3, 5, or 10 mL total volume.

[0068] Enrichment can occur using one or more types of separation modules. Several different modules are described herein, all of which can be fluidly coupled with one another in the series for enhanced performance.

[0069] In some embodiments, enrichment occurs by selective lysis as described above.

[0070] In one embodiment, enrichment of rare cells occurs using one or more size-based separation modules. Examples of size-based separation modules include filtration modules, sieves, matrixes, etc. Examples of size-based separation modules contemplated by the present invention include those disclosed in International Publication No. WO 2004/113877. Other size based separation modules are disclosed in International Publication No. WO 2004/0144651.

[0071] In some embodiments, a size-based separation module comprises one or more arrays of obstacles forming a network of gaps. The obstacles are configured to direct particles as they flow through the array/network of gaps into different directions or outlets based on the particle's hydrodynamic size. For example, as a blood sample flows through an array of obstacles, nucleated cells or cells having a hydrodynamic size larger than a predetermined certain size such as a cuttoff or predetermined size, e.g., 8 microns, are directed to a first outlet located on the opposite side of the array of obstacles from the fluid flow inlet, while the enucleated cells or cells having a hydrodynamic size smaller than a predetermined size, e.g., 8 microns, are directed to a second outlet also located on the opposite side of the array of obstacles from the fluid flow inlet.

[0072] An array can be configured to separate cells smaller or larger than a predetermined size by adjusting the size of the gaps, obstacles, and offset in the period between each successive row of obstacles. For example, in some embodiments, obstacles or gaps between obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170, or 200 microns in length or about 2, 4, 6, 8 or 10 microns in length. In some embodiments, an array for size-based separation includes more than 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000 obstacles that are arranged into more than 10, 20, 50, 100, 200, 500, or 1000 rows. Preferably, obstacles in a first row of obstacles are offset from a previous (upstream) row of obstacles by up to 50% the period of the previous row of obstacles. In some embodiments, obstacles in a first row of obstacles are offset from a previous row of obstacles by up to 45, 40, 35, 30, 25, 20, 15 or 10% the period of the previous row of obstacles. Furthermore, the distance between a first row of obstacles and a second row of obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170 or 200 microns. A particular offset can be continuous (repeating for multiple rows) or non-continuous. In some embodiments, a separation module includes multiple discrete arrays of obstacles fluidly coupled such that they are in series with one another. Each array of obstacles has a continuous offset. But each subsequent (downstream) array of obstacles has an offset that is different from the previous (upstream) offset. Preferably, each subsequent array of obstacles has a smaller offset that the previous array of obstacles. This allows for a refinement in the separation process as cells migrate through the array of obstacles. Thus, a plurality of arrays can be fluidly coupled in series or in parallel, (e.g., more than 2, 4, 6, 8, 10, 20, 30, 40, 50). Fluidly coupling separation modules (e.g., arrays) in parallel allows for high-throughput analysis of the sample, such that at least 1, 2, 5, 10, 20, 50, 100, 200, or 500

5

mL per hour flows through the enrichment modules or at least 1, 5, 10, or 50 million cells per hour are sorted or flow through the device.

[0073] FIG. 1A illustrates an example of a size-based separation module. Obstacles (which may be of any shape) are coupled to a flat substrate to form an array of gaps. A transparent cover or lid may be used to cover the array. The obstacles form a two-dimensional array with each successive row shifted horizontally with respect to the previous row of obstacles, where the array of obstacles directs component having a hydrodynamic size smaller than a predetermined size in a first direction and component having a hydrodynamic size larger that a predetermined size in a second direction. For enriching epithelial or circulating tumor cells from enucleated, the predetermined size of an array of obstacles can be get at 6-12 µm or 6-8 µm. For enriching fetal cells from a mixed sample (e.g. maternal blood sample) the predetermined size of an array of obstacles can be between 4-10 µm or 6-8 µm. The flow of sample into the array of obstacles can be aligned at a small angle (flow angle) with respect to a line-of-sight of the array. Optionally, the array is coupled to an infusion pump to perfuse the sample through the obstacles. The flow conditions of the size-based separation module described herein are such that cells are sorted by the array with minimal damage. This allows for downstream analysis of intact cells and intact nuclei to be more efficient and reliable.

[0074] In some embodiments, a size-based separation module comprises an array of obstacles configured to direct cells larger than a predetermined size to migrate along a line-of-sight within the array (e.g. towards a first outlet or bypass channel leading to a first outlet), while directing cells and analytes smaller than a predetermined size to migrate through the array of obstacles in a different direction than the larger cells (e.g. towards a second outlet). Such embodiments are illustrated in part in FIGS. 1B-1D.

[0075] A variety of enrichment protocols may be utilized although gentle handling of the cells is needed to reduce any mechanical damage to the cells or their DNA. This gentle handling also preserves the small number of fetal or rare cells in the sample. Integrity of the nucleic acid being evaluated is an important feature to permit the distinction between the genomic material from the fetal or rare cells and other cells in the sample. In particular, the enrichment and separation of the fetal or rare cells using the arrays of obstacles produces gentle treatment which minimizes cellular damage and maximizes nucleic acid integrity permitting exceptional levels of separation and the ability to subsequently utilize various formats to very accurately analyze the genome of the cells which are present in the sample in extremely low numbers.

[0076] In some embodiments, enrichment of rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells (CTCs)) occurs using one or more capture modules that selectively inhibit the mobility of one or more cells of interest. Preferable a capture module is fluidly coupled downstream to a size-based separation module. Capture modules can include a substrate having multiple obstacles that restrict the movement of cells or analytes greater than a predetermined size. Examples of capture modules that inhibit the migration of cells based on size are disclosed in U.S. Pat. Nos. 5,837,115 and 6,692,952.

[0077] In some embodiments, a capture module includes a two dimensional array of obstacles that selectively filters or captures cells or analytes having a hydrodynamic size greater than a particular gap size (predetermined size), International Publication No. WO 2004/113877.

[0078] In some cases a capture module captures analytes (e.g., cells of interest or not of interest) based on their affinity. For example, an affinity-based separation module that can capture cells or analytes can include an array of obstacles adapted for permitting sample flow through, but for the fact that the obstacles are covered with binding moieties that selectively bind one or more analytes (e.g., cell populations) of interest (e.g., red blood cells, fetal cells, epithelial cells or nucleated cells) or analytes not-of-interest (e.g., white blood cells). Arrays of obstacles adapted for separation by capture can include obstacles having one or more shapes and can be arranged in a uniform or non-uniform order. In some embodiments, a two-dimensional array of obstacles is staggered such that each subsequent row of obstacles is offset from the previous row of obstacles to increase the number of interactions between the analytes being sorted (separated) and the obstacles.

[0079] Binding moieties coupled to the obstacles can include e.g., proteins (e.g., ligands/receptors), nucleic acids having complementary counterparts in retained analytes, antibodies, etc. In some embodiments, an affinity-based separation module comprises a two-dimensional array of obstacles covered with one or more antibodies selected from the group consisting of: anti-CD71, anti-CD235a, anti-CD36, anti-carbohydrates, anti-selectin, anti-CD45, anti-GPA, anti-antigen-i, anti-EpCAM, anti-E-cadherin, and anti-Muc-1.

[0080] FIG. 2A illustrates a path of a first analyte through an array of posts wherein an analyte that does not specifically bind to a post continues to migrate through the array, while an analyte that does bind a post is captured by the array. FIG. 2B is a picture of antibody coated posts. FIG. 2C illustrates coupling of antibodies to a substrate (e.g., obstacles, side walls, etc.) as contemplated by the present invention. Examples of such affinity-based separation modules are described in International Publication No. WO 2004/029221.

[0081] In some embodiments, a capture module utilizes a magnetic field to separate and/or enrich one or more analytes (cells) based on a magnetic property or magnetic potential in such analyte of interest or an analyte not of interest. For example, red blood cells which are slightly diamagnetic (repelled by magnetic field) in physiological conditions can be made paramagnetic (attributed by magnetic field) by deoxygenation of the hemoglobin into methemoglobin. This magnetic property can be achieved through physical or chemical treatment of the red blood cells. Thus, a sample containing one or more red blood cells and one or more white blood cells can be enriched for the red blood cells by first inducing a magnetic property in the red blood cells and then separating the red blood cells from the white blood cells by flowing the sample through a magnetic field (uniform or non-uniform).

[0082] For example, a maternal blood sample can flow first through a size-based separation module to remove enucleated cells and cellular components (e.g., analytes having a hydrodynamic size less than 6 µms) based on size. Subsequently, the enriched nucleated cells (e.g., analytes having a hydrodynamic size greater than 6 µms) white blood cells and nucleated red blood cells are treated with a reagent, such as CO_2, N_2, or $NaNO_2$, that changes the magnetic property of the red blood cells' hemoglobin. The treated sample then flows through a magnetic field (e.g., a column coupled to an external magnet), such that the paramagnetic analytes (e.g., red blood cells) will be captured by the magnetic field while the

white blood cells and any other non-red blood cells will flow through the device to result in a sample enriched in nucleated red blood cells (including fetal nucleated red blood cells or fnRBC's). Additional examples of magnetic separation modules are described in U.S. application Ser. No. 11/323,971, filed Dec. 29, 2005 entitled "Devices and Methods for Magnetic Enrichment of Cells and Other Particles" and U.S. application Ser. No. 11/227,904, filed Sep. 15, 2005, entitled "Devices and Methods for Enrichment and Alteration of Cells and Other Particles".

[0083] Subsequent enrichment steps can be used to separate the rare cells (e.g. fnRBC's) from the non-rare cells maternal nucleated red blood cells. In some embodiments, a sample enriched by size-based separation followed by affinity/magnetic separation is further enriched for rare cells using fluorescence activated cell sorting (FACS) or selective lysis of a subset of the cells.

[0084] In some embodiments, enrichment involves detection and/or isolation of rare cells or rare DNA (e.g. fetal cells or fetal DNA) by selectively initiating apoptosis in the rare cells. This can be accomplished, for example, by subjecting a sample that includes rare cells (e.g. a mixed sample) to hyperbaric pressure (increased levels of CO_2; e.g. 4% CO_2). This will selectively initiate apoptosis in the rare or fragile cells in the sample (e.g. fetal cells). Once the rare cells (e.g. fetal cells) begin apoptosis, their nuclei will condense and optionally be ejected from the rare cells. At that point, the rare cells or nuclei can be detected using any technique known in the art to detect condensed nuclei, including DNA gel electrophoresis, in situ labeling of DNA nick using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick labeling (TUNEL) (Gavrieli, Y., et al. J. Cell Biol. 119:493-501 (1992)), and ligation of DNA strand breaks having one or two-base 3' overhangs (Taq polymerase-based in situ ligation). (Didenko V., et al. J. Cell Biol. 135:1369-76 (1996)).

[0085] In some embodiments ejected nuclei can further be detected using a size based separation module adapted to selectively enrich nuclei and other analytes smaller than a predetermined size (e.g. 6 microns) and isolate them from cells and analytes having a hydrodynamic diameter larger than 6 microns. Thus, in one embodiment, the present invention contemplated detecting fetal cells/fetal DNA and optionally using such fetal DNA to diagnose or prognose a condition in a fetus. Such detection and diagnosis can occur by obtaining a blood sample from the female pregnant with the fetus, enriching the sample for cells and analytes larger than 8 microns using, for example, an array of obstacles adapted for size-base separation where the predetermined size of the separation is 8 microns (e.g. the gap between obstacles is up to 8 microns). Then, the enriched product is further enriched for red blood cells (RBC's) by oxidizing the sample to make the hemoglobin puramagnetic and flowing the sample through one or more magnetic regions. This selectively captures the RBC's and removes other cells (e.g. white blood cells) from the sample. Subsequently, the fnRBC's can be enriched from mnRBC's in the second enriched product by subjecting the second enriched product to hyperbaric pressure or other stimulus that selectively causes the fetal cells to begin apoptosis and condense/eject their nuclei. Such condensed nuclei are then identified/isolated using e.g. laser capture microdissection or a size based separation module that separates components smaller than 3, 4, 5 or 6 microns from a sample. Such fetal nuclei can then by analyzed using any method known in the art or described herein.

[0086] In some embodiments, when the analyte desired to be separated (e.g., red blood cells or white blood cells) is not ferromagnetic or does not have a potential magnetic property, a magnetic particle (e.g., a bead) or compound (e.g., Fe^{3+}) can be coupled to the analyte to give it a magnetic property. In some embodiments, a bead coupled to an antibody that selectively binds to an analyte of interest can be decorated with an antibody elected from the group of anti CD71 or CD75. In some embodiments a magnetic compound, such as Fe^{3+}, can be couple to an antibody such as those described above. The magnetic particles or magnetic antibodies herein may be coupled to any one or more of the devices herein prior to contact with a sample or may be mixed with the sample prior to delivery of the sample to the device(s). Magnetic particles can also be used to decorate one or more analytes (cells of interest or not of interest) to increase the size prior to performing size-based separation.

[0087] Magnetic field used to separate analytes/cells in any of the embodiments herein can uniform or non-uniform as well as external or internal to the device(s) herein. An external magnetic field is one whose source is outside a device herein (e.g., container, channel, obstacles). An internal magnetic field is one whose source is within a device contemplated herein. An example of an internal magnetic field is one where magnetic particles may be attached to obstacles present in the device (or manipulated to create obstacles) to increase surface area for analytes to interact with to increase the likelihood of binding. Analytes captured by a magnetic field can be released by demagnetizing the magnetic regions retaining the magnetic particles. For selective release of analytes from regions, the demagnetization can be limited to selected obstacles or regions. For example, the magnetic field can be designed to be electromagnetic, enabling turn-on and turn-off off the magnetic fields for each individual region or obstacle at will.

[0088] FIG. 3 illustrates an embodiment of a device configured for capture and isolation of cells expressing the transferrin receptor from a complex mixture. Monoclonal antibodies to CD71 receptor are readily available off-the-shelf and can be covalently coupled to magnetic materials comprising any conventional ferroparticles, such as, but not limited to ferrous doped polystyrene and ferroparticles or ferro-colloids (e.g., from Miltenyi and Dynal). The anti CD71 bound to magnetic particles is flowed into the device. The antibody coated particles are drawn to the obstacles (e.g., posts), floor, and walls and are retained by the strength of the magnetic field interaction between the particles and the magnetic field. The particles between the obstacles and those loosely retained with the sphere of influence of the local magnetic fields away from the obstacles are removed by a rinse.

[0089] One or more of the enrichment modules herein (e.g., size-based separation module(s) and capture module(s)) may be fluidly coupled in series or in parallel with one another. For example a first outlet from a separation module can be fluidly coupled to a capture module. In some embodiments, the separation module and capture module are integrated such that a plurality of obstacles acts both to deflect certain analytes according to size and direct them in a path different than the direction of analyte(s) of interest, and also as a capture module to capture, retain, or bind certain analytes based on size, affinity, magnetism or other physical property.

[0090] In any of the embodiments herein, the enrichment steps performed have a specificity and/or sensitivity greater than 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3,

7

99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or 99.95% The retention rate of the enrichment module(s) herein is such that≧50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9% of the analytes or cells of interest (e.g., nucleated cells or nucleated red blood cells or nucleated from red blood cells) are retained. Simultaneously, the enrichment modules are configured to remove≧50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9% of all unwanted analytes (e.g., red blood-platelet enriched cells) from a sample.

[0091] Any of the enrichment methods herein may be further supplemented by splitting the enriched sample into aliquots or sub-samples. In some embodiments, an enriched sample is split into at least 2, 5, 10, 20, 50, 100, 200, 500, or 1000 sub-samples. Thus when an enriched sample comprises about 500 cells and is split into 500 or 1000 different sub-samples, each sub-sample will have 1 or 0 cells.

[0092] In some cases a sample is split or arranged such that each sub-sample is in a unique or distinct location (e.g. well). Such location may be addressable. Each site can further comprise a capture mechanism to capture cell(s) to the site of interest and/or release mechanism for selectively releasing cells from the cite of interest. In some cases, the well is configured to hold a single cell.

[0093] III. Sample Analysis

[0094] In some embodiments, the methods herein are used for detecting the presence or conditions of rare cells that are in a mixed sample (optionally even after enrichment) at a concentration of up to 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or 1% of all cells in the mixed sample, or at a concentration of less than 1:2, 1:4, 1:10, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:1,000,000, 1:2,000,000, 1:5,000,000, 1:10,000,000, 1:20,000,000, 1:50,000,000 or 1:100,000,000 of all cells in the sample, or at a concentration of less than 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, or 1×10^{-7} cells/ μL of a fluid sample. In some embodiments, the mixed sample has a total of up to 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or 100 rare cells (e.g. fetal cells or epithelial cells).

[0095] Enriched target cells (e.g., fnRBC) may be "binned" prior to further analysis of the enriched cells (FIGS. 34 & 35). Binning is any process which results in the reduction of complexity and/or total cell number of the enriched cell output. Binning may be performed by any method known in the art or described herein. One method of binning is by serial dilution. Such dilution may be carried out using any appropriate platform (e.g., PCR wells, microtiter plates) and appropriate buffers. Other methods include nanofluidic systems which can separate samples into droplets (e.g., BioTrove, Raindance, Fluidigm). Such nanofluidic systems may result in the presence of a single cell present in a nanodroplet.

[0096] Binning may be preceded by positive selection for target cells including, but not limited to, affinity binding (e.g. using anti-CD71 antibodies). Alternately, negative selection of non-target cells may precede binning. For example, output from a size-based separation module may be passed through a magnetic hemoglobin enrichment module (MHEM) which selectively removes WBCs from the enriched sample by attracting magnetized hemoglobin-containing cells.

[0097] For example, the possible cellular content of output from enriched maternal blood which has been passed through a size-based separation module (with or without further enrichment by passing the enriched sample through a MHEM) may consist of: 1) approximately 20 fnRBC; 2) 1,500 mnRBC; 3) 4,000-40,000 WBC; 4) 15×10^6 RBC. If this

sample is separated into 100 bins (PCR wells or other acceptable binning platform), each bin would be expected to contain: 1) 80 negative bins and 20 bins positive for one fnRBC; 2) 150 mnRBC; 3) 400-4,000 WBC; 4) 15×10^4 RBC. If separated into 10,000 bins, each bin would be expected to contain: 1) 9,980 negative bins and 20 bins positive for one fnRBC; 2) 8,500 negative bins and 1,500 bins positive for one mnRBC; 3)<1-4 WBC; 4) 15×10^2 RBC. One of skill in the art will recognize that the number of bins may be increased or decreased depending on experimental design and/or the platform used for binning. Reduced complexity of the binned cell populations may facilitate further genetic and/or cellular analysis of the target cells by reducing the number of non-target bins in an individual bin.

[0098] Analysis may be performed on individual bins to confirm the presence of target cells (e.g. fnRBC) in the individual bin. Such analysis may consist of any method known in the art including, but not limited to, FISH, PCR, STR detection, SNP analysis, biomarker detection, and sequence analysis (FIGS. 34 & 35).

[0099] For example, a peripheral maternal venous blood sample enriched by the methods herein can be analyzed to determine pregnancy or a condition of a fetus (e.g., sex of fetus or aneuploidy). The analysis step for fetal cells may further involves comparing the ratio of maternal to paternal genomic DNA on the identified fetal cells.

[0100] IV. Fetal Biomarkers

[0101] In some embodiments fetal biomarkers may be used to detect and/or isolate fetal cells, after enrichment or after detection of fetal abnormality or lack thereof. For example, this may be performed by distinguishing between fetal and maternal nRBCs based on relative expression of a gene (e.g., DYS1, DYZ, CD-71, ε- and ζ-globin) that is differentially expressed during fetal development. In preferred embodiments, biomarker genes are differentially expressed in the first and/or second trimester. "Differentially expressed," as applied to nucleotide sequences or polypeptide sequences in a cell or cell nuclei, refers to differences in over/under-expression of that sequence when compared to the level of expression of the same sequence in another sample, a control or a reference sample. In some embodiments, expression differences can be temporal and/or cell-specific. For example, for cell-specific expression of biomarkers, differential expression of one or more biomarkers in the cell(s) of interest can be higher or lower relative to background cell populations. Detection of such difference in expression of the biomarker may indicate the presence of a rare cell (e.g., fnRBC) versus other cells in a mixed sample (e.g., background cell populations). In other embodiments, a ratio of two or more such biomarkers that are differentially expressed can be measured and used to detect rare cells.

[0102] In one embodiment, fetal biomarkers comprise differentially expressed hemoglobins. Erythroblasts (nRBCs) are very abundant in the early fetal circulation, virtually absent in normal adult blood and by having a short finite lifespan, there is no risk of obtaining fnRBC which may persist from a previous pregnancy. Furthermore, unlike trophoblast cells, fetal erythroblasts are not prone to mosaic characteristics.

[0103] Yolk sac erythroblasts synthesize ε-, ζ-, γ- and α-globins, these combine to form the embryonic hemoglobins. Between six and eight weeks, the primary site of erythropoiesis shifts from the yolk sac to the liver, the three embryonic hemoglobins are replaced by fetal hemoglobin (HbF) as

the predominant oxygen transport system, and α- and ζ-globin production gives way to γ-, α- and β-globin production within definitive erythrocytes (Peschle et al., 1985). HbF remains the principal hemoglobin until birth, when the second globin switch occurs and β-globin production accelerates.

[0104] Hemoglobin (Hb) is a heterodimer composed of two identical a globin chains and two copies of a second globin. Due to differential gene expression during fetal development, the composition of the second chain changes from α globin during early embryonic development (1 to 4 weeks of gestation) to γ globin during fetal development (6 to 8 weeks of gestation) to β globin in neonates and adults as illustrated in (Table 1).

TABLE 1

Relative expression of ε, γ and β in maternal and fetal RBCs.

		ε	γ	B
1st trimester	Fetal	++	++	–
	Maternal	–	+/–	++
2nd trimester	Fetal	–	++	+/–
	Maternal	–	+/–	++

[0105] In the late-first trimester, the earliest time that fetal cells may be sampled by CVS, fnRBCs contain, in addition to α globin, primarily ε and γ globin. In the early to mid second trimester, when amniocentesis is typically performed, fnR-BCs contain primarily γ globin with some adult β globin. Maternal cells contain almost exclusively α and β globin, with traces of γ detectable in some samples. Therefore, by measuring the relative expression of the ε, γ and β genes in RBCs purified from maternal blood samples, the presence of fetal cells in the sample can be determined. Furthermore, positive controls can be utilized to assess failure of the FISH analysis itself.

[0106] In various embodiments, fetal cells are distinguished from maternal cells based on the differential expression of hemoglobins β, γ or ε. Expression levels or RNA levels can be determined in the cytoplasm or in the nucleus of cells. Thus in some embodiments, the methods herein involve determining levels of messenger RNA (mRNA), ribosomal RNA (rRNA), or nuclear RNA (nRNA).

[0107] In some embodiments, identification of fnRBCs can be achieved by measuring the levels of at least two hemoglobins in the cytoplasm or nucleus of a cell. In various embodiments, identification and assay is from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 fetal nuclei. Furthermore, total nuclei arrayed on one or more slides can number from about 100, 200, 300, 400, 500, 700, 800, 5000, 10,000, 100,000, 1,000,000, 2,000,000 to about 3,000,000. In some embodiments, a ratio for γ/β or ε/β is used to determine the presence of fetal cells, where a number less than one indicates that a fnRBC(s) is not present. In some embodiments, the relative expression of γ/β or ε/β provides a fnRBC index ("FNI"), as measured by γ or ε relative to β. In some embodiments, a FNI for γ/β greater than 5, 10, 15, 20, 25, 30, 35, 40, 45, 90, 180, 360, 720, 975, 1020, 1024, 1250 to about 1250, indicate that a fnRBC(s) is present. In yet other embodiments, a FNI for γ/β of less than about 1 indicates that a fnRBC(s) is not present. Preferably, the above FNI is determined from a sample obtained during a first trimester. However, similar ratios can be used during second trimester and third trimester.

[0108] In some embodiments, the expression levels are determined by measuring nuclear RNA transcripts including, nascent or unprocessed transcripts. In another embodiment, expression levels are determined by measuring mRNA, including ribosomal RNA. There are many methods known in the art for imaging (e.g., measuring) nucleic acids or RNA including, but not limited to, using expression arrays from Affymetrix, Inc. or Illumina, Inc.

[0109] RT-PCR primers can be designed by targeting the globin variable regions, selecting the amplicon size, and adjusting the primers annealing temperature to achieve equal PCR amplification efficiency. Thus TaqMan probes can be designed for each of the amplicons with well-separated fluorescent dyes, Alexa fluor®-355 for ε, Alexa Fluor®-488 for γ, and Alexa Fluor-555 for β. The specificity of these primers can be first verified using ε, γ, and β cDNA as templates. The primer sets that give the best specificity can be selected for further assay development. As an alternative, the primers can be selected from two exons spanning an intron sequence to amplify only the mRNA to eliminate the genomic DNA contamination.

[0110] The primers selected can be tested first in a duplex format to verify their specificity, limit of detection, and amplification efficiency using target cDNA templates. The best combinations of primers can be further tested in a triplex format for its amplification efficiency, detection dynamic range, and limit of detection.

[0111] Various commercially available reagents are available for RT-PCR, such as One-step RT-PCR reagents, including Qiagen One-Step RT-PCR Kit and Applied Biosytems TaqMan One-Step RT-PCR Master Mix Reagents kit. Such reagents can be used to establish the expression ratio of ε, γ, and β using purified RNA from enriched samples. Forward primers can be labeled for each of the targets, using Alexa fluor-355 for ε, Alexa fluor-488 for γ, and Alexa fluor-555 for β. Enriched cells can be deposited by cytospinning onto glass slides. Additionally, cytospinning the enriched cells can be performed after in situ RT-PCR. Thereafter, the presence of the fluorescent-labeled amplicons can be visualized by fluorescence microscopy. The reverse transcription time and PCR cycles can be optimized to maximize the amplicon signal:background ratio to have maximal separation of fetal over maternal signature. Preferably, signal:background ratio is greater than 5, 10, 50 or 100 and the overall cell loss during the process is less than 50, 10 or 5%.

[0112] V. Fetal Cell Analysis

[0113] FIG. 4 illustrates an overview of some embodiments of the present invention.

[0114] Aneuploidy means the condition of having less than or more than the normal diploid number of chromosomes. In other words, it is any deviation from euploidy. Aneuploidy includes conditions such as monosomy (the presence of only one chromosome of a pair in a cell's nucleus), trisomy (having three chromosomes of a particular type in a cell's nucleus), tetrasomy (having four chromosomes of a particular type in a cell's nucleus), pentasomy (having five chromosomes of a particular type in a cell's nucleus), triploidy (having three of every chromosome in a cell's nucleus), and tetraploidy (having four of every chromosome in a cell's nucleus). Birth of a live triploid is extraordinarily rare and such individuals are quite abnormal, however triploidy occurs in about 2-3% of all human pregnancies and appears to be a factor in about 15% of

all miscarriages. Tetraploidy occurs in approximately 8% of all miscarriages. (http://www.emedicine.com/med/topic3241.htm).

[0115] In step **400**, a sample is obtained from an animal, such as a human. In some embodiments, animal or human is pregnant, suspected of being pregnant, or may have been pregnant, and, the systems and methods herein are used to diagnose pregnancy and/or conditions of the fetus (e.g. trisomy). In some embodiments, the animal or human is suspected of having a condition, has a condition, or had a condition (e.g., cancer) and, the systems and methods herein are used to diagnose the condition, determine appropriate therapy, and/or monitor for recurrence.

[0116] In both scenarios a sample obtained from the animal can be a blood sample e.g., of up to 50, 40, 30, 20, or 15 mL. In some cases multiple samples are obtained from the same animal at different points in time (e.g. before therapy, during therapy, and after therapy, or during 1st trimester, 2nd trimester, and 3rd trimester of pregnancy).

[0117] In optional step **402**, rare cells (e.g., fetal cells or epithelial cells) or DNA of such rare cells are enriched using one or more methods known in the art or described herein. For example, to enrich fetal cells from a maternal blood sample, the sample can be applied to a size-base separation module (e.g., two-dimensional array of obstacles) configured to direct cells or particles in the sample greater than 8 microns to a first outlet and cells or particles in the sample smaller than 8 microns to a second outlet. The fetal cells can subsequently be further enriched from maternal white blood cells (which are also greater than 8 microns) based on their potential magnetic property. For example, N_2 or anti-CD71 coated magnetic beads is added to the first enriched product to make the hemoglobin in the red blood cells (maternal and fetal) paramagnetic. The enriched sample is then flowed through a column coupled to an external magnet. This captures both the fnRBC's and mnRBC's creating a second enriched product. The sample can then be subjected to hyperbaric pressure or other stimulus to initiate apoptosis in the fetal cells. Fetal cells/nuclei can then be enriched using microdissection, for example. It should be noted that even an enriched product can be dominated (>50%) by cells not of interest (e.g. maternal red blood cells). In some cases an enriched sample has the rare cells (or rare genomes) consisting of up to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, or 50% of all cells (or genomes) in the enriched sample. For example, using the systems herein, a maternal blood sample of 20 mL from a pregnant human can be enriched for fetal cells such that the enriched sample has a total of about 500 cells, 2% of which are fetal and the rest are maternal.

[0118] In step **404**, the enriched product is split between two or more discrete locations. In some embodiments, a sample is split into at least 2, 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3,000, 4,000, 5000, or 10,000 total different discrete sites or about 100, 200, 500, 1000, 1200, 1500 sites. In some embodiments, output from an enrichment module is serially divided into wells of a 1536 microwell plate (FIG. **8**). This can result in one cell or genome per location or 0 or 1 cell or genome per location. In some embodiments, cell splitting results in more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10,000, 20,000, 50,000, 100,000, 200,000, or 500,000 cells or genomes per location. When splitting a sample enriched for epithelial cells, endothelial cells, or CTC's, the load at each discrete location (e.g., well) can include several leukocytes, while one only some of the loads includes one or more CTC's. When splitting a sample enriched for fetal cells preferably each site includes 0 or 1 fetal cells.

[0119] Examples of discrete locations which could be used as addressable locations include, but are not limited to, wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles, or in-situ within a cell or nuclear membrane. In some embodiments, the discrete cells are addressable such that one can correlate a cell or cell sample with a particular location.

[0120] Examples of methods for splitting a sample into discrete addressable locations include, but are not limited to, fluorescent activated cell sorting (FACS) (Sherlock, J V et al. Ann. Hum. Genet. 62 (Pt. 1): 9-23 (1998)), micromanipulation (Samura, O., Ct al Hum. Genet. 107(1):28-32 (2000)) and dilution strategies (Findlay, I. et al. Mol. Cell. Endocrinol. 183 Suppl 1:S5-12 (2001)). Other methods for sample splitting cell sorting and splitting methods known in the art may also be used. For example, samples can be split by affinity sorting techniques using affinity agents (e.g. antibodies) bound to any immobilized or mobilized substrate (Samura O., et al., Hum. Genet. 107(1):28-32 (2000)). Such affinity agents can be specific to a cell type e.g. RBC's fetal cells epithelial cells including those specifically binding EpCAM, antigen-i, or CD-71.

[0121] In some embodiments, a sample or enriched sample is transferred to a cell sorting device that includes an array of discrete locations for capturing cells traveling along a fluid flow. The discrete locations can be arranged in a defined pattern across a surface such that the discrete sites are also addressable. In some embodiments, the sorting device is coupled to any of the enrichment devices known in the art or disclosed herein. Examples of cell sorting devices included are described in International Publication No. WO 01/35071. Examples of surfaces that may be used for creating arrays of cells in discrete addressable sites include, but are not limited to, cellulose, cellulose acetate, nitrocellulose, glass, quartz or other crystalline substrates such as gallium arsenide, silicones, metals, semiconductors, various plastics and plastic copolymers, cyclo-olefin polymers, various membranes and gels, microspheres, beads and paramagnetic or supramagnetic microparticles.

[0122] In some embodiments, a sorting device comprises an array of wells or discrete locations wherein each well or discrete location is configured to hold up to 1 cell. Each well or discrete addressable location may have a capture mechanism adapted for retention of such cell (e.g. gravity, suction, etc.) and optionally a release mechanism for selectively releasing a cell of interest from a specific well or site (e.g. bubble actuation). Figure B illustrates such an embodiment.

[0123] In step **406**, nucleic acids of interest from each cell or nuclei arrayed are tagged by amplification. Preferably, the amplified/tagged nucleic acids include at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 90, 90 or 100 polymorphic genomic DNA regions such as short tandem repeats (STRs) or variable number of tandem repeats ("VNTR"). When the amplified DNA regions include one or more STR/s/, the STR/s/ are selected for high heterozygosity (variety of alleles) such that the paternal allele of any fetal cell is more likely to be distinct in length from the maternal allele. This results in improved power to detect the presence of fetal cells in a mixed sample and any potential of fetal abnormalities in such cells. In some embodiment, STR(s) amplified are selected for their association with a particular condition. For example, to deter-

mine fetal abnormality an STR sequence comprising a mutation associated with fetal abnormality or condition is amplified. Examples of STRs that can be amplified/analyzed by the methods herein include, but are not limited to D21S1414, D21S1411, D21S1412, D21S11 MBP, D13S634, D13S631, D18S535, AmgXY and XHPRT. Additional STRs that can be amplified/analyzed by the methods herein include, but are not limited to, those at locus F13B (1:q31-q32); TPOX (2:p23-2pter); FIBRA (FGA) (4:q28); CSFIPO (5:q33.3-q34); F13A (6:p24-p25); THOI (11:p15-15.5); VWA (12:p12-pter); CDU (12p12-pter); D14S1434 (14:q32.13); CYAR04 (p450) (15: q21.1) D21S11 (21:q11-q21) and D22S1045 (22:q12.3). In some cases, STR loci are chosen on a chromosome suspected of trisomy and on a control chromosome. Examples of chromosomes that are often trisomic include chromosomes 21, 18, 13, and X. In some cases, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 STRs are amplified per chromosome tested (Samura, O. et al., Clin. Chem. 47(9):1622-6 (2001)). For example amplification can be used to generate amplicons of up to 20, up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 150, up to 200, up to 300, up to 400, up to 500 or up to 1000 nucleotides in length. Di-, tri-, tetra-, or penta-nucleotide repeat STR loci can be used in the methods described herein.

[0124] To amplify and tag genomic DNA region(s) of interest, PCR primers can include: (i) a primer element, (ii) a sequencing element, and (iii) a locator element.

[0125] The primer element is configured to amplify the genomic DNA region of interest (e.g. STR). The primer element includes, when necessary, the upstream and downstream primers for the amplification reactions. Primer elements can be chosen which are multiplexible with other primer pairs from other tags in the same amplification reaction (e.g. fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primer element can have at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40 or 50 nucleotide bases, which are designed to specifically hybridize with and amplify the genomic DNA region of interest.

[0126] The sequencing element can be located on the 5' end of each primer element or nucleic acid tag. The sequencing element is adapted to cloning and/or sequencing of the amplicons. (Marguiles, M, Nature 437 (7057): 376-80) The sequencing element can be about 4, 6, 8, 10, 18, 20, 28, 36, 46 or 50 nucleotide bases in length.

[0127] The locator element (also known as a unique tag sequence), which is often incorporated into the middle part of the upstream primer, can include a short DNA or nucleic acid sequence between 4-20 by in length (e.g., about 4, 6, 8, 10, or 20 nucleotide bases). The locator element makes it possible to pool the amplicons from all discrete addressable locations following the amplification step and analyze the amplicons in parallel. In some embodiments each locator element is specific for a single addressable location.

[0128] Tags are added to the cells/DNA at each discrete location using an amplification reaction. Amplification can be performed using PCR or by a variety of methods including, but not limited to, singleplex PCR, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start PCR, nested PCR, in situ polonony PCR, in situ rolling circle amplification

(RCA), bridge PCR, picotiter PCR, multiple strand displacement amplification (MDA), and emulsion PCR. Other suitable amplification methods include the ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR) and nucleic acid based sequence amplification (NABSA). Additional examples of amplification techniques using PCR primers are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and 6,582,938.

[0129] In some embodiments, a further PCR amplification is performed using nested primers for the one or more genomic DNA regions of interest to ensure optimal performance of the multiplex amplification. The nested PCR amplification generates sufficient genomic DNA starting material for further analysis such as in the parallel sequencing procedures below.

[0130] In step 408, genomic DNA regions tagged/amplified are pooled and purified prior to further processing. Methods for pooling and purifying genomic DNA are known in the art.

[0131] In step 410, pooled genomic DNA/amplicons are analyzed to measure, e.g. allele abundance of genomic DNA regions (e.g. STRs amplified). In some embodiments such analysis involves the use of capillary gel electrophoresis (CGE). In other embodiments, such analysis involves sequencing or ultra deep sequencing.

[0132] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[0133] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the target nucleic acid sequence. Sequence-by-synthesis can be initiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immediately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incorporation of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Publication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100, 000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[0134] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase, apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next,

deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

[0135] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins. Methods to image sequence information after performing ligation are known in the art.

[0136] Preferably, analysis involves the use of ultra-deep sequencing, such as described in Marguiles et al., Nature 437 (7057): 376-80 (2005). Briefly, the amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence which all remain bound to the bead. Such amplification can occur by PCR. Each bead can be placed in a separate well, which can be a (optionally addressable) picolitre-sized well. In some embodiments, each bead is captured within a droplet of a PCR-reaction-mixture-in-oil-emulsion and PCR amplification occurs within each droplet. The amplification on the bead results in each bead carrying at least one million, at least 5 million, or at least 10 million copies of the original amplicon coupled to it. Finally, the beads are placed into a highly parallel sequencing by synthesis machine which generates over 400,000 reads (~100 bp per read) in a single 4 hour run.

[0137] Other methods for ultra-deep sequencing that can be used are described in Hong, S. et al. Nat. Biotechnol. 22(4): 435-9 (2004); Bennett, B. et al. Pharmacogenomics 6(4):373-82 (2005); Shendure, P. et al. Science 309 (5741):1728-32 (2005).

[0138] The role of the ultra-deep sequencing is to provide an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs, the error rates of the multiplex PCR, and the Poisson sampling statistics associated with the sequencing procedures.

[0139] In one example, the enrichment output from step 402 results in approximately 500 cells of which 98% are maternal cells and 2% are fetal cells. Such enriched cells are subsequently split into 500 discrete locations (e.g., wells) in a microtiter plate such that each well contains 1 cell. PCR is used to amplify STR's (~3-10 STR loci) on each chromosome of interest. Based on the above example, as the fetal/maternal ratio goes down, the aneuploidy signal becomes diluted and more loci are needed to average out measurement errors asso-

ciated with variable DNA amplification efficiencies from locus to locus. The sample division into wells containing ~1 cell proposed in the methods described herein achieves pure or highly enriched fetal/maternal ratios in some wells, alleviating the requirements for averaging of PCR errors over many loci.

[0140] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor $1+f/2$. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the $\ln(ratio(m/p))$, where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of $\ln(ratio(m/p))$ between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a 2(\sigma_{allele}^2)/N_c \qquad (1)$$

[0141] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[0142] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \qquad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \qquad (4)$$

[0143] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in the following Table 2 for various values of σ_{allele} and f.

TABLE 2

Required number of loci per chromosome as a function of σ_{allele} and f.

| | f | | |
σ_{allele}	0.1	0.3	1.0
0.1	144	16	1
0.3	1296	144	13
1.0	14400	1600	144

Since sample splitting decreases the number of starting genome copies which increases σ_{allele} at the same time that it increases the value of f in some wells, the methods herein are based on the assumption that the overall effect of splitting is favorable; i.e., that the PCR errors do not increase too fast with decreasing starting number of genome copies to offset the benefit of having some wells with large f. The required number of loci can be somewhat larger because for many loci the paternal allele is not distinct from the maternal alleles, and this incidence depends on the heterozygosity of the loci. In the case of highly polymorphic STRs, this amounts to an approximate doubling of N.

[0144] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of ampli-

cons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error σ_{allele}. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele})^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by

$$N_{reads}=288\,N_{wells}f \qquad (5)$$

[0145] When performing sample splitting, a rough approximation is to stipulate that the sample splitting causes f to approach unity in at least a few wells. If the sample splitting is to have advantages, then it must be these wells which dominate the information content in the final result. Therefore, Equation (5) with f=1 is adopted, which suggests a minimum of about 300 reads per well. For 500 wells, this gives a minimum requirement for ~150,000 sequence reads. Allowing for the limited heterozygosity of the loci tends to increase the requirements (by a factor of ~2 in the case of STRs), while the effect of reinforcement of data from multiple wells tends to relax the requirements with respect to this result (in the baseline case examined above it is assumed that ~10 wells have a pure fetal cell). Thus the required total number of reads per patient is expected to be in the range 100,000-300,000.

[0146] In step 412, wells with rare cells/alleles (e.g., fetal alleles) are identified. The locator elements of each tag can be used to sort the reads (~200,000 sequence reads) into 'bins' which correspond to the individual wells of the microtiter plates (~500 bins). The sequence reads from each of the bins (~400 reads per bin) are then separated into the different genomic DNA region groups, (e.g. STR loci,) using standard sequence alignment algorithms. The aligned sequences from each of the bins are used to identify rare (e.g., non-maternal) alleles. It is estimated that on average a 15 ml blood sample from a pregnant human will result in ~10 bins having a single fetal cell each.

[0147] The following are two examples by which rare alleles can be identified. In a first approach, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above in order to obtain maternal alleles. This sample can be a white blood cell fraction or simply a dilution of the original sample before enrichment. In a second approach, the sequences or genotypes for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles determines which discrete location (e.g. well) contained fetal cells. Determining the number of bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original cell population or enriched sample. Bins containing fetal cells are identified with high levels of confidence because the non-maternal alleles are detected by multiple independent polymorphic DNA regions, e.g. STR loci.

[0148] In step 414, condition of rare cells or DNA is determined. This can be accomplished by determining abundance of selected alleles (polymorphic genomic DNA regions) in

bin(s) with rare cells/DNA. In some embodiments, allele abundance is used to determine aneuploidy, e.g. chromosomes 13, 18 and 21. Abundance of alleles can be determined by comparing ratio of maternal to paternal alleles for each genomic region amplified (e.g., ~12 STR's). For example, if 12 STRs are analyzed, for each bin there are 33 sequence reads for each of the STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker will be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). (Adinolfi, P. et al., Prenat. Diagn, 17(13): 1299-311 (1997)). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[0149] In some embodiments allele abundance is used to determine segmental anuepolidy. Normal diploid cells have two copies of each chromosome and thus two alleles of each gene or loci. Changes in the allele abundance for a particular chromosomal region may be indicative of a chromosomal rearrangement, such as a deletion, duplication or translocation event. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given segmental aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[0150] The determination of fetal trisomy can be used to diagnose conditions such as abnormal fetal genotypes, including, trisomy 13, trisomy 18, trisomy 21 (Down syndrome) and Klinefelter Syndrome (XXY). Other examples of abnormal fetal genotypes include, but are not limited to, aneuploidy such as, monosomy of one or more chromosomes (X chromosome monosomy, also known as Turner's syndrome), trisomy of one or more chromosomes (13, 18, 21, and X), tetrasomy and pentasomy of one or more chromosomes (which in humans is most commonly observed in the sex chromosomes, e.g. XXXX, XXYY, XXXY, XYYY, XXXXX, XXXXY, XXXYY, XYYYY and XXYYY), triploidy (three of every chromosome, e.g. 69 chromosomes in humans), tetraploidy (four of every chromosome, e.g. 92 chromosomes in humans) and multiploidy. In some embodiments, an abnormal fetal genotype is a segmental aneuploidy. Examples of segmental aneuploidy include, but are not limited to, 1p36 duplication, dup(17)(p11.2p11.2) syndrome, Down syndrome, Pelizaeus-Merzbacher disease, dup(22) (q11.2q11.2) syndrome, and cat-eye syndrome. In some cases, an abnormal fetal genotype is due to one or more deletions of sex or autosomal chromosomes, which may result in a condition such as Cri-du-chat syndrome, Wolf-Hirschhorn, Williams-Beuren syndrome, Charcot-Marie-Tooth disease, Hereditary neuropathy with liability to pressure palsies. Smith-Magenis syndrome, Neurofibromatosis, Alagille syndrome, Velocardiofacial syndrome, DiGeorge syndrome, Steroid sulfatase deficiency, Kallmann syndrome, Microphthalmia with linear skin defects, Adrenal hypoplasia, Glycerol kinase deficiency, Pelizaeus-Merzbacher disease, Testis-determining factor on Y, Azoospermia (factor a),

Azoospermia (factor b), Azoospermia (factor c), or 1p36 deletion. In some embodiments, a decrease in chromosomal number results in an XO syndrome.

[0151] In one embodiment, the methods of the invention allow for the determination of maternal or paternal trisomy. In some embodiments, the methods of the invention allow for the determination of trisomy or other conditions in fetal cells in a mixed maternal sample arising from more than one fetus.

[0152] In another aspect of the invention, standard quantitative genotyping technology is used to declare the presence of fetal cells and to determine the copy numbers (ploidies) of the fetal chromosomes. Several groups have demonstrated that quantitative genotyping approaches can be used to detect copy number changes (Wang, Moorhead et al. 2005). However, these approaches do not perform well on mixtures of cells and typically require a relatively large number of input cells (~10,000). The current invention addresses the complexity issue by performing the quantitative genotyping reactions on individual cells. In addition, multiplex PCR and DNA tags are used to perform the thousands of genotyping reaction on single cells in highly parallel fashion.

[0153] An overview of this embodiment is illustrated in FIG. 5.

[0154] In step 500, a sample (e.g., a mixed sample of rare and non-rare cells) is obtained from an animal or a human. See, e.g., step 400 of FIG. 4. Preferably, the sample is a peripheral maternal blood sample.

[0155] In step 502, the sample is enriched for rare cells (e.g., fetal cells) by any method known in the art or described herein. See, e.g., step 402 of FIG. 4.

[0156] In step 504, the enriched product is split into multiple distinct sites (e.g., wells). See, e.g., step 404 of FIG. 4.

[0157] In step 506, PCR primer pairs for amplifying multiple (e.g., 2-100) highly polymorphic genomic DNA regions (e.g., SNPs) are added to each discrete site or well in the array or microtiter plate. For example, PCR primer pairs for amplifying SNPs along chromosome 13, 18, 21 and/or X can be designed to detect the most frequent aneuoploidies. Other PCR primer pairs can be designed to amplify SNPs along control regions of the genome where aneuploidy is not expected. The genomic loci (e.g., SNPs) in the aneuploidy region or aneuploidy suspect region are selected for high polymorphism such that the paternal alleles of the fetal cells are more likely to be distinct from the maternal alleles. This improves the power to detect the presence of fetal cells in a mixed sample as well as fetal conditions or abnormalities. SNPs can also be selected for their association with a particular condition to be detected in a fetus. In some cases, one or more than one, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 SNPs are analyzed per target chromosome (e.g., 13, 18, 21, and/or X). The increase number of SNPs interrogated per chromosome ensures accurate results. PCR primers are chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers are designed to generate amplicons 10-200, 20-180, 40-160, 60-140 or 70-100 bp in size to increase the performance of the multiplex PCR.

[0158] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. The multiplex amplification of single cells is helpful to generate sufficient starting material for the parallel genotyping procedure. Multiplex PCT can be performed on single cells with minimal levels of allele dropout and preferential amplification. See Sherlock, J., et al. Ann. Hum. Genet. 61 (Pt 1): 9-23 (1998); and Findlay, I., et al. Mol. Cell. Endocrinol. 183 Suppl. 1: S5-12 (2001).

[0159] In step 508, amplified polymorphic DNA region(s) of interest (e.g., SNPs) are tagged e.g., with nucleic acid tags. Preferably, the nucleic acid tags serve two roles: to determine the identity of the different SNPs and to determine the identity of the bin from which the genotype was derived. Nucleic acid tags can comprise primers that allow for allele-specific amplification and/or detection. The nucleic acid tags can be of a variety of sizes including up to 10 base pairs, 10-40, 15-30, 18-25 or ~22 base pair long.

[0160] In some embodiments, a nucleic acid tag comprises a molecular inversion probe (MIP). Examples of MIPs and their uses are described in Hardenbol, P., et al., Nat. Biotechnol. 21(6):673-8 (2003); Hardenbol, P., et al., Genome Res. 15(2):269-75 (2005); and Wang, Y., et al., Nucleic Acids Res. 33(21):e183 (2005). FIG. 7A illustrates one example of a MIP assay used herein. The MIP tag can include a locator element to determine the identity of the bin from which the genotype was derived. For example, when output from an enrichment procedure results in about 500 cells, the enriched product/cells can be split into a microliter plate containing 500 wells such that each cell is in a different distinct well. FIG. 7B illustrates a microtiter plate with 500 wells each of which contains a single cell. Each cell is interrogated at 10 different SNPs per chromosome, on 4 chromosomes (e.g., chromosomes 13, 18, 21 and X). This analysis requires 40 MIPs per cell/well for a total of 20,000 tags per 500 wells (i.e., 4 chromosomes×10 SNPs×500 wells). The tagging step can also include amplification of the MIPs after their rearrangement or enzymatic "gap fill".

[0161] In one embodiment, a nucleic acid tag comprises a unique property, such as a difference in mass or chemical properties from other tags. In another embodiments a nucleic acid tag comprises a photoactivatable label, so that it crosslinks where it binds. In another embodiment a nucleic acid tag can be used as a linker for ultra deep sequencing. In another embodiment a nucleic acid tag can be used as a linker for arrays. In another embodiment a nucleic acid tag comprises a unique fluorescent label, (Such as FAM, JOE, ROX, NED, HEX, SYBR, PET, TAMRA, VIC, CY-3, CY-5, dR6G, DS-33, LIZ, DS-02, dR110, and Texas Red) which can be used to differentiate individual DNA fragments. In another embodiment a nucleic acid tag can serve as primer or hybridization site for a probe, to facilitate signal amplification or detection from a single cell by using a tractable marker. In some embodiments the labeled nucleic acid tag can be analyzed using a system coupled to a light source, such as an ABI 377, 310, 3700 or any other system which can detect fluorescently labeled DNA.

[0162] In step 510, the tagged amplicons are pooled together for further analysis.

[0163] In step 512, the genotype at each polymorphic site is determined and/or quantified using any technique known in the art. In one embodiment, genotyping occurs by hybridization of the MIP tags to a microarray containing probes complementary to the sequences of each MIP tag. See U.S. Pat. No. 6,858,412.

[0164] Using the example described above with the MIP probes, the 20,000 tags are hybridized to a single tag array containing complementary sequences to each of the tagged MIP probes. Microarrays (e.g. tag arrays) can include a plu-

rality of nucleic acid probes immobilized to discrete spots (e.g., defined locations or assigned positions) on a substrate surface. For example, a microarray can have at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1,000, 5,000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, or 100,000 different probes complementary to MIP tagged probes. Methods to prepare microarrays capable to monitor several genes according to the methods of the invention are well known in the art. Examples of microarrays that can be used in nucleic acid analysis that may be used are described in U.S. Pat. No. 6,300,063, U.S. Pat. No. 5,837, 832, U.S. Pat. No. 6,969, 589, U.S. Pat. No. 6,040,138, U.S. Pat. No. 6,858, 412, US Publication No. 2005/0100893, US Publication No. 2004/0018491, US Publication No. 2003/ 0215821 and US Publication No. 2003/0207295.

[0165] In step **516**, bins with rare alleles (e.g., fetal alleles) are identified. Using the example described above, rare allele identification can be accomplished by first using the 22 bp tags to sort the 20,000 genotypes into 500 bins which correspond to the individual wells of the original microtiter plates. Then, one can identify bins containing non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of its provides an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles can be detected by 40 independent SNPS s which provide an extremely high level of confidence in the result.

[0166] In step **518**, a condition such as trisomy is determined based on the rare cell polymorphism. For example, after identifying the~10 bins that contain fetal cells, one can determine the ploidy of chromosomes 13, 18, 21 and X of such cells by comparing the ratio of maternal to paternal alleles for each of~10 SNPs on each chromosome (X, 13, 18, 21). The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the~10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[0167] As described above, an enriched maternal sample with 500 cells can be split into 500 discrete locations such that each location contains one cell. If ten SNPs are analyzed in each of four different chromosomes, forty tagged MIP probes are added per discrete location to analyze forty different SNPs per cell. The forty SNPs are then amplified in each location using the primer element in the MIP probe as described above. All the amplicons from all the discrete locations are then pooled and analyzed using quantitative genotyping as describe above. In this example a total of 20,000 probes in a microarray are required to genotype the same 40 SNPs in each of the 500 discrete locations (4 chromosomes×10 SNPs×500 discrete locations).

[0168] The above embodiment can also be modified to provide for genotyping by hybridizing the nucleic acid tags to bead arrays as are commercially available by Illumina, Inc. and as described in U.S. Pat. Nos. 7,040,959; 7,035,740; 7,033,754; 7,025,935, 6,998,274; 6,942,968; 6,913,884; 6,890,764; 6,890,741; 6,858,394; 6,846,460; 6,812,005; 6,770,441; 6,663,832; 6,620,584; 6,544,732; 6,429,027; 6,396,995; 6,355,431 and US Publication Application Nos. 20060019258; 20050266432; 20050244870; 20050216207; 20050181394; 20050164246; 20040224353; 20040185482;

20030198573; 20030175773; 20030003490; 20020187515; and 20020177141; as well as Shen, R., et al. Mutation Research 573 70-82 (2005).

[0169] An overview of the use of nucleic acid tags is described in FIG. 7C. After enrichment and amplification as described above, target genomic DNA regions are activated in step **702** such that they may bind paramagnetic particles. In step **703** assay oligonucleotides, hybridization buffer, and paramagnetic particles are combined with the activated DNA and allowed to hybridize (hybridization step). In some cases, three oligonucleotides are added for each SNP to be detected. Two of the three oligos are specific for each of the two alleles at a SNP position and are referred to as Allele-Specific Oligos (ASOs). A third oligo hybridizes several bases downstream from the SNP site and is referred to as the Locus-Specific Oligo (LSO). All three oligos contain regions of genomic complementarity (C1, C2, and C3) and universal PCR primer sites (P1, P2 and P3). The LSO also contains a unique address sequence (Address) that targets a particular bead type. In some cases, up to 1,536 SNPs may be interrogated in this manner. During the primer hybridization process, the assay oligonucleotides hybridize to the genomic DNA sample bound to paramagnetic particles. Because hybridization occurs prior to any amplification steps, no amplification bias is introduced into the assay. The above primers can further be modified to serve the two roles of determining the identity of the different SNPs and to determining the identity of the bin from which the genotype was derived. In step **704**, following the hybridization step, several wash steps are performed reducing noise by removing excess and mis-hybridized oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. In step **705**, the joined, full-length products provide a template for performing PCR reactions using universal PCR primers P1, P2, and P3. Universal primers P1 and P2 are labeled with two different labels (e.g., Cy3 and Cy5). Other labels that can be used include, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, scattering or fluorescent nanoparticles, Raman signal generating moieties, or electrochemical detection moieties. In step **706**, the single-stranded, labeled DNAs are eluted and prepared for hybridization. In step **707**, the single-stranded, labeled DNAs are hybridized to their complement bead type through their unique address sequence. Hybridization of the GoldenGate Assay products onto the Array Matrix of Beadchip allows for separation of the assay products in solution, onto a solid surface for individual SNP genotype readout. In step **708**, the array is washed and dried. In step **709**, a reader such as the BeadArray Reader is used to analyze signals from the label. For example, when the labels are dye labels such as Cy3 and Cy5, the reader can analyze the fluorescence signal on the Sentrix Array Matrix or BeadChip. In step **710**, a computer readable medium having a computer executable logic recorded on it can be used in a computer to perform receive data from one or more quantified DNA genomic regions to automate genotyping clusters and callings. Expression detection and analysis using microarrays is described in part in Valk, P. J. et al. New England Journal of Medicine 350(16), 1617-28, 2004; Modlich, O. et al. Clinical Cancer Research 10(10), 3410-21, 2004; Onken, Michael D. et al. Cancer Res. 64(20), 7205-7209, 2004; Gardian, et al. J. Biol. Chem. 280(1), 556-563, 2005; Becker, M. et al. Mol.

Cancer Ther. 4(1), 151-170, 2005; and Flechner, S M et al. Am J Transplant 4(9), 1475-89, 2004; as well as in U.S. Pat. Nos. 5,445,934; 5,700,637; 5,744,305; 5,945,334; 6,054,270; 6,140,044; 6,261,776; 6,291,183; 6,346,413; 6,399,365; 6,420,169; 6,551,817; 6,610,482; 6,733,977; and EP 619 321; 323 203.

[0170] In any of the embodiments herein, preferably, more than 1000, 5,000, 10,000, 50,000, 100,000, 500,000, or 1,000,000 SNPs are interrogated in parallel.

[0171] In another aspect of the invention, illustrated in part by FIG. 6, the systems and methods herein can be used to diagnose, prognose, and monitor neoplastic conditions such as cancer in a patient. Examples of neoplastic conditions contemplated herein include acute lymphoblastic leukemia, acute or chronic lymphocyctic or granulocytic tumor, acute myeloid leukemia, acute promyelocytic leukemia, adenocarcinoma, adenoma, adrenal cancer, basal cell carcinoma, bone cancer, brain cancer, breast cancer, bronchi cancer, cervical dysplasia, chronic myelogenous leukemia, colon cancer, epidermoid carcinoma, Ewing's sarcoma, gallbladder cancer, gallstone tumor, giant cell tumor, glioblastoma multiforma, hairy-cell tumor, head cancer, hyperplasia, hyperplastic comeal nerve tumor, in situ carcinoma, intestinal ganglion-euroma, islet cell tumor, Kaposi's sarcoma, kidney cancer, larynx cancer, leiomyomater tumor, liver cancer, lung cancer, lymphomas, malignant carcinoid, malignant hypercalcemia, malignant melanomas, marfanoid habitus tumor, medullary carcinoma, metastatic skin carcinoma, mucosal neuromas, mycosis fungoide, myelodysplastic syndrome, myeloma, neck cancer, neural tissue cancer, neuroblastoma, osteogenic sarcoma, osteosarcoma, ovarian tumor, pancreas cancer, parathyroid cancer, pheochromocytoma, polycythemia vera, primary brain tumor, prostate cancer, rectum cancer, renal cell tumor, retinoblastoma, rhabdomyosarcoma, seminoma, skin cancer, small-cell lung tumor, soft tissue sarcoma, squamous cell carcinoma, stomach cancer, thyroid cancer, topical skin lesion, veticulum cell sarcoma, and Wilm's tumor.

[0172] Cancers such as breast, colon, liver, ovary, prostate, and lung as well as other tumors exfoliate epithelial cells into the bloodstream. The presence of an increased number epithelial cells is associated with an active tumor or other neoplastic condition, tumor progression and spread, poor response to therapy, relapse of disease, and/or decreased survival over a period of several years. Therefore, enumerating and/or analyzing epithelial cells and CTC's in the bloodstream can be used to diagnose, prognose, and/or monitor neoplastic conditions.

[0173] In step 600, a sample is obtained from an animal such as a human. The human can be suspected of having cancer or cancer recurrence or may have cancer and is in need of therapy selection. The sample obtained is a mixed sample comprising normal cells as well as one or more CTCs, epithelial cells, endothelial cells, stem cells, or other cells indicative of cancer. In some cases, the sample is a blood sample. In some cases multiple samples are obtained from the animal at different points in time (e.g., regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[0174] In step 602, the mixed sample is then enriched for epithelial cells or CTC's or other cell indicative of cancer. Epithelial cells that are exfoliated from solid tumors have been found in very low concentrations in the circulation of patients with advanced cancers of the breast, colon, liver, ovary, prostate, and lung, and the presence or relative number

of these cells in blood has been correlated with overall prognosis and response to therapy. These epithelial cells which are in fact CTCs can be used as an early indicator of tumor expansion or metastasis before the appearance of clinical symptoms.

[0175] CTCs are generally larger than most blood cells. Therefore, one useful approach for obtaining CTCs in blood is to enrich them based on size, resulting in a cell population enriched in CTCs. Another way to enrich CTCs is by affinity separation, using antibodies specific for particular cell surface markers may be used. Useful endothelial cell surface markers include CD105, CD106, CD144, and CD146; useful tumor endothelial cell surface markers include TEM1, TEM5, and TEM8 (see, e.g., Carson-Walter et al., Cancer Res. 61:6649-6655 (2001)); and useful mesenchymal cell surface markers include CD 133. Antibodies to these or other markers may be obtained from, e.g., Chemicon, Abcam, and R & D Systems.

[0176] In one example, a size-based separation module that enriches CTC's from a fluid sample (e.g., blood) comprises an array of obstacles that selectively deflect particles having a hydrodynamic size larger than 10 μm into a first outlet and particles having a hydrodynamic size smaller than 10 μm into a second outlet is used to enrich epithelial cells and CTC's from the sample.

[0177] In step 603, the enriched product is split into a plurality of discrete sites, such as mirowells. Examplary microwells that can be used in the present invention include microplates having 1536 wells as well as those of lesser density (e.g., 96 and 384 wells). Microwell plate design contemplated herein include those have 14 outputs that can be automatically dispensed at the same time, as well as those with 16, 24, or 32 outputs such that e.g., 32 outputs can be dispenses simultaneously. FIG. 9 illustrates one embodiments of a microwell plate contemplated herein.

[0178] Dispensing of the cells into the various discrete sites is preferably automated. In some cases, about 1, 5, 10, or 15 μL of enriched sample is dispensed into each well. Preferably, the size of the well and volume dispensed into each well is such that only 1 cell is dispensed per well and only 1-5 or less than 3 cells can fit in each well.

[0179] An exemplary array for sample splitting is illustrated in FIG. 8A. FIG. 8B illustrates an isometric view and FIG. 8B illustrates a top view and cross sectional view of such an array. A square array of wells is arranged such that each subsequent row or column of wells is identical to the previous row or column of wells, respectively. In some embodiments, an array of wells is configured in a substrate or plate that about 2.0 cm^2, 2.5 cm^2, 3 cm^2 or larger. The wells can be of any shape, e.g., round, square, or oval. The height or width of each well can be between 5-50 μm, 10-40 μm, or about 25 μm, or about 25 μm. The depth of each well can be up to 100, 80, 60, or 40 μm; and the radius between the centers of two wells in one column is between 10-60 μm, 20-50 μm, or about 35 μm. Using these configurations, an array of wells of area 2.5 cm^2 can have a at least 0.1×10^6 wells, 0.2×10^6 wells, 0.3×10^6 wells, 0.4×10^6 wells, or 0.5×10^6 wells.

[0180] In some embodiments, such as those illustrated in FIG. 8C each well may have an opening at the bottom. The bottom opening is preferably smaller in size than the cells of interest. In this case, if the average radius of a CTC is about 10 μm, the bottom opening of each well can have a radius of up to 8, 7, 6, 5, 4, 3, 2 or 1 μm. The bottom opening allows for cells non-of interest and other components smaller than the

cell of interest to be removed from the well using flow pressure, leaving the cells of interest behind in the well for further processing. Methods and systems for actuating removal of cells from discrete predetermined sites are disclosed in U.S. Pat. No. 6,692,952 and U.S. application Ser. No. 11/146,581.

[0181] In some cases, the array of wells can be a micro-electro-mechanical system (MEMS) such that it integrates mechanical elements, sensors, actuators, and electronics on a common silicon substrate through microfabrication technology. Any electronics in the system can be fabricated using integrated circuit (IC) process sequences (e.g., CMOS, Bipolar, or BICMOS processes), while the micromechanical components are fabricated using compatible micromachining processes that selectively etch away parts of the silicon wafer or add new structural layers to form the mechanical and electromechanical devices. One example of a MEMS array of wells includes a MEMS isolation element within each well. The MEMS isolation element can create a flow using pressure and/or vacuum to increase pressure on cells and particles not of interest to escape the well through the well opening. In any of the embodiments herein, the array of wells can be coupled to a microscope slide or other substrate that allows for convenient and rapid optical scanning of all chambers (i.e. discrete sites) under a microscope. In some embodiments, a 1536-well microtiter plate is used for enhanced convenience of reagent addition and other manipulations.

[0182] In some cases, the enriched product can be split into wells such that each well is loaded with a plurality of leukocytes (e.g., more than 100, 200, 500, 1000, 2000, or 5000). In some cases, about 2500 leukocytes are dispensed per well, while random wells will have a single epithelial CTC or up to 2, 3, 4, or 5 epithelial cells or CTC's. Preferably, the probability of getting a single epithelial cell or CTC into a well is calculated such that no more than 1 CTC is loaded per well. The probability of dispensing CTC's from a sample into wells can be calculated using Poisson statistics. When dispensing a 15 mL sample into 1536 wellplate at 10 μL per well, it is not until the number of CTC's in the sample is>100 that there is more than negligible probability of two or more CTC's being loaded into the sample well. FIG. 9 illustrates the probability density function of loading two CTC's into the same plate.

[0183] In step 604, rare cells (e.g. epithelial cells or CTC's) or rare DNA is detected and/or analyzed in each well.

[0184] In some embodiments, detection and analysis includes enumerating epithelial cells and/or CTC's. CTCs typically have a short half-life of approximately one day, and their presence generally indicates a recent influx from a proliferating tumor. Therefore, CTCs represent a dynamic process that may reflect the current clinical status of patient disease and therapeutic response. Thus, in some embodiments, step 604 involves enumerating CTC and/or epithelial cells in a sample (array of wells) and determining based on their number if a patient has cancer, severity of condition, therapy to be used, or effectiveness of therapy administered.

[0185] In some cases, the method herein involve making a series of measurements, optionally made at regular intervals such as one day, two days, three days, one week, two weeks, one month, two months, three months, six months, or one year, one may track the level of epithelial cells present in a patient's bloodstream as a function of time. In the case of existing cancer patients, this provides a useful indication of the progression of the disease and assists medical practitioners in making appropriate therapeutic choices based on the increase, decrease, or lack of change in epithelial cells, e.g.,

CTCs, in the patient's bloodstream. For those at risk of cancer, a sudden increase in the number of cells detected may provide an early warning that the patient has developed a tumor. This early diagnosis, coupled with subsequent therapeutic intervention, is likely to result in an improved patient outcome in comparison to an absence of diagnostic information.

[0186] In some cases, more than one type of cell (e.g., epithelial, endothelial, etc.) can be enumerated and a determination of a ratio of numbers of cells or profile of various cells can be obtained to generate the diagnosis or prognosis.

[0187] Alternatively, detection of rare cells or rare DNA (e.g. epithelial cells or CTC's) can be made by detecting one or more cancer biomarkers, e.g., any of those listed in FIG. 10 in one or more cells in the array. Detection of cancer biomarkers can be accomplished using, e.g., an antibody specific to the marker or by detecting a nucleic acid encoding a cancer biomarker, e.g., listed in FIG. 9.

[0188] In some cases single cell analysis techniques are used to analyze individual cells in each well. For example, single cell PCR may be performed on a single cell in a discrete location to detect one or more mutant alleles in the cell (Thornhill A R, J. Mol. Diag; (4) 11-29 (2002)) or a mutation in a gene listed in FIG. 9. In-cell PCR, gene expression analysis can be performed even when the number of cells per well is very low (e.g. 1 cell per well) using techniques known in the art. (Giordano et al., Am. J. Pathol. 159:1231-1238 (2001), and Buckhaults et al., Cancer Res. 63:4144-4149 (2003). In some cases, single cell expression analysis can be performed to detection expression of one or more genes of interest (Liss B., Nucleic Acids Res., 30 (2002)) including those listed in FIG. 9. Furthermore, ultra-deep sequencing can be performed on single cells using methods such as those described in Marguiles M., et al. Nature, "Genome sequencing in microfabricated high-density picolitre reactors." DOI 10.1038, in which whole genomes are fragmented, fragments are captured using common adapters on their own beads and within droplets of an emulsion, clonally amplified. Such ultra-deep sequencing can also be used to detect mutations in genes associated with cancer, such as those listed in FIG. 9. In addition, fluorescence in-situ hybridization can be used, e.g., to determine the tissue or tissues of origin of the cells being analyzed.

[0189] In some cases, morphological analyses are performed on the cells in each well. Morphological analyses include identification, quantification and characterization of mitochondrial DNA, telomerase, or nuclear matrix proteins. Parrella et al., Cancer Res. 61:7623-7626 (2001); Jones et al., Cancer Res. 61:1299-1304 (2001); Fliss et al., Science 287: 2017-2019 (2000); and Soria et al., Clin. Cancer Res. 5:971-975 (1999). In particular, in some cases, the molecular analyses involves determining whether any mitochrondial abnormalities or whether perinuclear compartments are present. Carew et al., Mol. Cancer 1:9 (2002); and Wallace, Science 283:1482-1488 (1999).

[0190] A variety of cellular characteristics may be measured using any technique known in the art, including: protein phosphorylation, protein glycosylation, DNA methylation (Das et al., J. Clin. Oncol. 22:4632-4642 (2004)), microRNA levels (He et al., Nature 435:828-833 (2005), Lu et al., Nature 435:834-838 (2005), O'Donnell et al., Nature 435:839-843 (2005), and Calin et al., N. Engl. J. Med. 353:1793-1801 (2005)), cell morphology or other structural characteristics, e.g., pleomorphisms, adhesion, migration, binding, division,

level of gene expression, and presence of a somatic mutation. This analysis may be performed on any number of cells, including a single cell of interest, e.g., a cancer cell.

[0191]   In one embodiment, the cell(s) (such as fetal, maternal, epithelial or CTCs) in each well are lysed and RNA is extracted using any means known in the art. For example, The Quiagen RNeasy™ 96 bioRobot™ 8000 system can be used to automate high-throughput isolation of total RNA from each discrete site. Once the RNA is extracted reverse transcriptase reactions can be performed to generate cDNA sequences, which can then be used for performing multiplex PCR reactions on target genes. For example, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 target genes can be amplified in the same reaction. When more than one target genes are used in the same amplification reaction, primers are chosen to be multiplexable (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis) with other pairs of primers. Multiple dyes and multi-color fluorescence readout may be used to increase the multiplexing capacity. Examples of dyes that can be used to label primers for amplification include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties.

[0192]   In another embodiment, fetal or maternal cells or nuclei are enriched using one or more methods disclosed herein. Preferably, fetal cells are enriched by flowing the sample through an array of obstacles that selectively directs particles or cells of different hydrodynamic sizes into different outlets such that fetal cells and cells larger than fetal cells are directed into a first outlet and one or more cells or particles smaller than the rare cells are directed into a second outlet.

[0193]   Total RNA or poly-A mRNA is then obtained from enriched cell(s) (fetal or maternal cells) using purification techniques known in the art. Generally, about 1 μg-2 μg of total RNA is sufficient. Next, a first-strand complementary DNA (cDNA) is synthesized using reverse transcriptase and a single T7-oligo(dT) primer. Next, a second-strand cDNA is synthesized using DNA ligase, DNA polymerase, and RNase enzyme. Next, the double stranded cDNA (ds-cDNA) is purified.

[0194]   In another embodiment, total RNA is extracted from enriched cells (fetal cells or maternal cells). Next a, two one-quarter scale Message Amp II reactions (Ambion, Austin, Tex.) are performed for each RNA extraction using 200 ng of total RNA. MessageAmp is a procedure based on antisense RNA (aRNA) amplification, and involves a series of enzymatic reactions resulting in linear amplification of exceedingly small amounts of RNA for use in array analysis. Unlike exponential RNA amplification methods, such as NASBA and RT-PCR, aRNA amplification maintains representation of the starting mRNA population. The procedure begins with total or poly(A) RNA that is reverse transcribed using a primer containing both oligo(dT) and a T7 RNA polymerase promoter sequence. After first-strand synthesis, the reaction is treated with RNase H to cleave the mRNA into small fragments. These small RNA fragments serve as primers during a second-strand synthesis reaction that produces a double-stranded cDNA template.

[0195]   In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a fetal cell (such as by the presence of Y chromosome specific transcripts) or whether the fetal cell has a genetic abnormality (such as aneuploidy, abundance or type of alternative transcripts or problems with DNA methylation or imprinting).

[0196]   In one embodiment, PCR amplification can be performed on genes that are expressed in epithelial cells and not in normal cells, e.g., white blood cells or other cells remaining in an enriched product. Exemplary genes that can be analyzed according to the methods herein include EGFR, EpCAM, GA733-2, MUC-1, HER-2, Claudin-7 and any other gene identified in FIG. 10.

[0197]   For example, analysis of the expression level or pattern of such a polypeptide or nucleic acid, e.g., cell surface markers, genomic DNA, mRNA, or microRNA, may result in a diagnosis or prognosis of cancer.

[0198]   In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a fetal cell (such as by the presence of Y chromosome specific transcripts) or whether the fetal cell has a genetic abnormality (such as anueploidy, or problems with DNA methylation or imprinting).

[0199]   In some embodiments, analysis step 604 involves identifying cells from a mixed sample that express genes which are not expressed in the non-rare cells (e.g. EGFR or EpCAM). For example, an important indicator for circulating tumor cells is the presence/expression of EGFR or EGF at high levels wherein non-cancerous epithelial cells will express EGFR or EGF at smaller amounts if at all.

[0200]   In addition, for lung cancer and other cancers, the presence or absence of certain mutations in EGFR can be associated with diagnosis and/or prognosis of the cancer as well and can also be used to select a more effective treatment (see, e.g., International Publication WO 2005/094357). For example, many non-small cell lung tumors with EGFR mutations respond to small molecule EGFR inhibitors, such as gefitinib (Iressa; AstraZeneca), but often eventually acquire secondary mutations that make them drug resistant. In some embodiments, one can determine a therapy treatment for a patient by enriching epithelial cells and/or CTC's using the methods herein, splitting sample of cells (preferably so no more than 1 CTC is in a discrete location), and detecting one or more mutations in the EGFR gene of such cells. Exemplary mutations that can be analyzed include those clustered around the ATP-binding pocket of the EGFR TK domain, which are known to make cells susceptible to gefitinib inhibition. Thus, presence of such mutations supports a diagnosis of cancer that is likely to respond to treatment using gefitinib.

[0201]   Many patients who respond to gefitinib eventually develop a second mutation, often a methionine-to-threonine substitution at position 790 in exon 20 of the TK domain. This type of mutation renders such patients resistant to gefitinib. Therefore, the present invention contemplates testing for this mutation as well to provide further diagnostic information.

[0202]   Since many EGFR mutations, including all EGFR mutations in NSC lung cancer reported to date that are known to confer sensitivity or resistance to gefitinib, lie within the coding regions of exons 18 to 21, this region of the EGFR gene may be emphasized in the development of assays for the presence of mutations. Examples of primers that can be used to detect mutations in EGFR include those listed in FIG. 11.

[0203] In step **605**, a determination is made as to the condition of a patient based on analysis made above. In some cases the patient can be diagnosed with cancer or lack thereof. In some cases, the patient can be prognosed with a particular type of cancer. In cases where the patient has cancer, therapy may be determined based on the types of mutations detected.

[0204] In another embodiment, cancer cells may be detected in a mixed sample (e.g. circulating tumor cells and circulating normal cells) using one or more of the sequencing methods described herein. Briefly, RNA is extracted from cells in each location and converted to cDNA as described above. Target genes are then amplified and high throughput ultra deep sequencing is performed to detect a mutation expression level associated with cancer.

[0205] VI. Computer Executable Logic

[0206] Any of the steps herein can be performed using computer program product that comprises a computer executable logic recorded on a computer readable medium. For example, the computer program can use data from target genomic DNA regions to determine the presence or absence of fetal cells in a sample and to determine fetal abnormalit (ies) in cells detected. In some embodiments, computer executable logic uses data input on STR or SNP intensities to determine the presence of fetal cells in a test sample and determine fetal abnormalities and/or conditions in said cells.

[0207] The computer program may be specially designed and configured to support and execute some or all of the functions for determining the presence of rare cells such as fetal cells or epithelial/CTC's in a mixed sample and abnormalities and/or conditions associated with such rare cells or their DNA including the acts of (i) controlling the splitting or sorting of cells or DNA into discrete locations (ii) amplifying one or more regions of genomic DNA e.g. trisomic region(s) and non-trisomic region(s) (particularly DNA polymorphisms such as STR and SNP) in cells from a mixed sample and optionally control sample, (iii) receiving data from the one or more genomic DNA regions analyzed (e.g. sequencing or genotyping data); (iv) identifying bins with rare (e.g. non-maternal) alleles, (v) identifying bins with rare (e.g. non-maternal) alleles as bins containing fetal cells or epithelial cells, (vi) determining number of rare cells (e.g. fetal cells or epithelial cells) in the mixed sample, (vii) detecting the levels of maternal and non-maternal alleles in identified fetal cells, (viii) detecting a fetal abnormality or condition in said fetal cells and/or (ix) detecting a neoplastic condition and information concerning such condition such as its prevalence, origin, susceptibility to drug treatment(s), etc. In particular, the program can fit data of the quantity of allele abundance for each polymorphism into one or more data models. One example of a data model provides for a determination of the presence or absence of aneuploidy using data of amplified polymorphisms present at loci in DNA from samples that are highly enriched for fetal cells. The determination of presence of fetal cells in the mixed sample and fetal abnormalities and/or conditions in said cells can be made by the computer program or by a user.

[0208] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor $1+f/2$. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the $\ln(ratio(m/p))$,

where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of $\ln(ratio(m/p))$ between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a + 2)/N_c \qquad (1)$$

[0209] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[0210] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \qquad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \qquad (4)$$

[0211] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in Table 2 for various values of σ_{allele} and f.

[0212] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of amplicons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error $_{allele}$. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele}^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by $N_{reads} = 288 N_{wells} f^3$. Thus, the program can determine the total number of reads that need to be obtained for determining the presence or absence of aneuploidy in a patient sample.

[0213] The computer program can work in any computer that may be any of a variety of types of general-purpose computers such as a personal computer, network server, workstation, or other computer platform now or later developed. In some embodiments, a computer program product is described comprising a computer usable medium having the computer executable logic (computer software program, including program code) stored therein. The computer executable logic can be executed by a processor, causing the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[0214] In one embodiment, the computer executing the computer logic of the invention may also include a digital input device such as a scanner. The digital input device can provide an image of the target genomic DNA regions (e.g. DNA polymorphism, preferably STRs or SNPs) according to method of the invention. For instance, the scanner can provide an image by detecting fluorescent, radioactive, or other emissions; by detecting transmitted, reflected, or scattered radiation; by detecting electromagnetic properties or characteristics; or by other techniques. Various detection schemes are employed depending on the type of emissions and other fac-

tors. The data typically are stored in a memory device, such as the system memory described above, in the form of a data file.

[0215] In one embodiment, the scanner may identify one or more labeled targets. For instance, in the genotyping analysis described herein a first DNA polymorphism may be labeled with a first dye that fluoresces at a particular characteristic frequency, or narrow band of frequencies, in response to an excitation source of a particular frequency. A second DNA polymorphisms may be labeled with a second dye that fluoresces at a different characteristic frequency. The excitation sources for the second dye may, but need not, have a different excitation frequency than the source that excites the first dye, e.g., the excitation sources could be the same, or different, lasers.

[0216] In one embodiment, a human being may inspect a printed or displayed image constructed from the data in an image file and may identify the data (e.g. fluorescence from microarray) that are suitable for analysis according to the method of the invention. In another embodiment, the information is provided in an automated, quantifiable, and repeatable way that is compatible with various image processing and/or analysis techniques.

[0217] Another aspect of the invention is kits which permit the enrichment and analysis of the rare cells present in small qualities in the samples. Such kits may include any materials or combination of materials described for the individual steps or the combination of steps ranging from the enrichment through the genetic analysis of the genomic material. Thus, the kits may include the arrays used for size-based separation or enrichment, labels for uniquely labeling each cell, the devices utilized for splitting the cells into individual addressable locations and the reagents for the genetic analysis. For example, a kit might contain the arrays for size-based separation, unique labels for the cells and reagents for detecting polymorphisms including STRs or SNPs, such as reagents for performing PCR.

[0218] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

EXAMPLES

Example 1

Separation of Fetal Cord Blood

[0219] FIG. 1E shows a schematic of the device used to separate nucleated cells from fetal cord blood.

[0220] Dimensions: 100 mm×28 mm×1 mm

[0221] Array design: 3 stages, gap size=18, 12 and 8 μm for the first, second and third stage, respectively.

[0222] Device fabrication: The arrays and channels were fabricated in silicon using standard photolithography and deep silicon reactive etching techniques. The etch depth is 140 μm. Through holes for fluid access are made using KOH wet etching. The silicon substrate was sealed on the etched face to form enclosed fluidic channels using a blood compatible pressure sensitive adhesive (9795, 3M, St Paul, Minn.).

[0223] Device packaging: The device was mechanically mated to a plastic manifold with external fluidic reservoirs to deliver blood and buffer to the device and extract the generated fractions.

[0224] Device operation: An external pressure source was used to apply a pressure of 2.0 PSI to the buffer and blood reservoirs to modulate fluidic delivery and extraction from the packaged device.

[0225] Experimental conditions: Human fetal cord blood was drawn into phosphate buffered saline containing Acid Citrate Dextrose anticoagulants. 1 mL of blood was processed at 3 mL/hr using the device described above at room temperature and within 48 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, Calif.) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, Mo.) and 2 mM EDTA (15575-020, Invitrogen, Carlsbad, Calif.).

[0226] Measurement techniques: Cell smears of the product and waste fractions (FIGS. 12A-12B) were prepared and stained with modified Wright-Giemsa (WG16, Sigma Aldrich, St. Louis, Mo.).

[0227] Performance: Fetal nucleated red blood cells were observed in the product fraction (FIG. 12A) and absent from the waste fraction (FIG. 12B).

Example 2

Isolation of Fetal Cells from Maternal Blood

[0228] The device and process described in detail in Example 1 were used in combination with immunomagnetic affinity enrichment techniques to demonstrate the feasibility of isolating fetal cells from maternal blood.

[0229] Experimental conditions: blood from consenting maternal donors carrying male fetuses was collected into K₂EDTA vacutainers (366643, Becton Dickinson, Franklin Lakes, N.J.) immediately following elective termination of pregnancy. The undiluted blood was processed using the device described in Example 1 at room temperature and within 9 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, Calif.) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, Mo.). Subsequently, the nucleated cell fraction was labeled with anti-CD71 microbeads (130-046-201, Miltenyi Biotech Inc., Auburn, Calif.) and enriched using the MiniMACS™ MS column (130-042-201, Miltenyi Biotech Inc., Auburn, Calif.) according to the manufacturer's specifications. Finally, the CD71-positive fraction was spotted onto glass slides.

[0230] Measurement techniques: Spotted slides were stained using fluorescence in situ hybridization (FISH) techniques according to the manufacturer's specifications using Vysis probes (Abbott Laboratories, Downer's Grove, IL). Samples were stained from the presence of X and Y chromosomes. In one case, a sample prepared from a known Trisomy 21 pregnancy was also stained for chromosome 21.

[0231] Performance: Isolation of fetal cells was confirmed by the reliable presence of male cells in the CD71-positive population prepared from the nucleated cell fractions (FIGS.

13A-13F). In the single abnormal case tested, the trisomy 21 pathology was also identified (FIG. **14**).

Example 3

Confirmation of the Presence of Male Fetal Cells in Enriched Samples

[0232] Confirmation of the presence of a male fetal cell in an enriched sample is performed using qPCR with primers specific for DYZ, a marker repeated in high copy number on the Y chromosome. After enrichment of fnRBC by any of the methods described herein, the resulting enriched fnRBC are binned by dividing the sample into 100 PCR wells. Prior to binning, enriched samples may be screened by FISH to determine the presence of any fnRBC containing an aneuploidy of interest. Because of the low number of fnRBC in maternal blood, only a portion of the wells will contain a single fnRBC (the other wells are expected to be negative for fnRBC). The cells are fixed in 2% Paraformaldehyde and stored at 4° C. Cells in each bin are pelleted and resuspended in 5 μl PBS plus 1 μl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65° C. for 60 minutes followed by inactivation of the Proteinase K by incubation for 15 minutes at 95° C. For each reaction, primer sets (DYZ forward primer TCGAGTGCATTCCATTCCG (SEQ ID NO: 57); DYZ reverse primer ATGGAATGGCATCAAACGGAA (SEQ ID NO: 58); and DYZ Taqman Probe 6FAM-TGGCTGTCCAT-TCCAMGBNFQ (SEQ ID NO: 59)), TaqMan Universal PCR master mix, No AmpErase and water are added. The samples are run and analysis is performed on an ABI 7300: 2 minutes at 50° C., 10 minutes 95° C. followed by 40 cycles of 95° C. (15 seconds) and 60° C. (1 minute). Following confirmation of the presence of male fetal cells, further analysis of bins containing fnRBC is performed. Positive bins may be pooled prior to further analysis.

[0233] FIG. 30 shows the results expected from such an experiment. The data in FIG. 30 was collected by the following protocol. Nucleated red blood cells were enriched from cord cell blood of a male fetus by sucrose gradient two Heme Extractions (HE). The cells were fixed in 2% paraformaldehyde and stored at 4° C. Approximately 10×1000 cells were pelleted and resuspended each in 5 μl PBS plus 1 μl 20 mg/ml Proteinase K (Sigma #P-2308). Cells were lysed by incubation at 65° C. for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95° C. Cells were combined and serially diluted 10-fold in PBS for 100, 10 and 1 cell per 6 μl final concentration were obtained. Six μl of each dilution was assayed in quadruplicate in 96 well format. For each reaction, primer sets (0.9 uM DYZ forward primer TCGAGTGCAT-TCCATTCCG (SEQ ID NO: 57); 0.9 uM DYZ reverse primer ATGGAATGGCATCAAACGGAA (SEQ ID NO: 58); and 0.5 uM DYZ TaqMan Probe 6FAM-TGGCTGTCCAT-TCCA-MGBNFQ (SEQ ID NO: 59)), TaqMan Universal PCR master mix, No AmpErase and water were added to a final volume of 25 μl per reaction. Plates were run and analyzed on an ABI 7300: 2 minutes at 50° C., 10 minutes 95° C. followed by 40 cycles of 95° C. (15 seconds) and 60° C. (1 minute). These results show that detection of a single fnRBC in a bin is possible using this method.

Example 4

Confirmation of the presence of fetal cells in enriched samples by STR analysis.

[0234] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then sub-

jected to FISH analysis using probes specific to the aneuploidy of interest (e.g., trisomy 13, trisomy 18, and XYY). Individual positive cells are isolated by "plucking" individual positive cells from the enhanced sample using standard micromanipulation techniques. Using a nested PCR protocol, STR marker sets are amplified and analyzed to confirm that the FISH-positive aneuploid cell(s) are of fetal origin. For this analysis, comparison to the maternal genotype is typical. An example of a potential resulting data set is shown in Table 3. Non-maternal alleles may be proven to be paternal alleles by paternal genotyping or genotyping of known fetal tissue samples. As can be seen, the presence of paternal alleles in the resulting cells, demonstrates that the cell is of fetal origin (cells # 1, 2, 9, and 10). Positive cells may be pooled for further analysis to diagnose aneuploidy of the fetus, or may be further analyzed individually.

TABLE 3

STR locus alleles in maternal and fetal cells

DNA Source	STR locus D14S	STR locus D16S	STR locus D8S	STR locus F13B	STR locus vWA
Maternal alleles	14, 17	11, 12	12, 14	9, 9	16, 17
Cell #1 alleles		8			19
Cell #2 alleles	17		15		
Cell #3 alleles			14		
Cell #4 alleles					
Cell #5 alleles	17	12		9	
Cell #6 alleles					
Cell #7 alleles					19
Cell #8 alleles					
Cell #9 alleles	17		14	7, 9	17, 19
Cell #10 alleles			15		

Example 5

Confirmation of the Presence of Fetal cells in Enriched Samples by SNP Analysis

[0235] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then subjected to FISH analysis using probes specific to the aneuploidy of interest (e.g., triploidy 13, triploidy 18, and XYY). Samples testing positive with FISH analysis are then binned into 96 microtiter wells, each well containing 15 μl of the enhanced sample. Of the 96 wells, 5-10 are expected to contain a single fnRBC and each well should contain approximately 1000 nucleated maternal cells (both WBC and mn RBC). Cells are pelleted and resuspended in 5 μl PBS plus 1 μl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65° C. for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95° C.

[0236] In this example, the maternal genotype (BB) and fetal genotype (AB) for a particular set of SNPs is known. The genotypes A and B encompass all three SNPs and differ from each other at all three SNPs. The following sequence from chromosome 7 contains these three SNPs (rs7795605, rs7795611 and rs7795233 indicated in brackets, respectively):

```
                                    (SEQ ID NO: 60)
(ATGCAGCAAGGCACAGACTAA[G/A]CAAGGAGA[G/C]

GCAAAATTTTC[A/G]TAGGGGAGAGAAATGGGTCATT.)
```

[0237] In the first round of PCR, genomic DNA from binned enriched cells is amplified using primers specific to the outer portion of the fetal-specific allele A and which flank the interior SNP (forward primer ATGCAGCAAGGCACA-GACTACG (SEQ ID NO: 61); reverse primer AGAGGG-GAGAGAAATGGGTCATT (SEQ ID NO: 62)). In the second round of PCR, amplification using real time SYBR Green PCR is performed with primers specific to the inner portion of allele A and which encompass the interior SNP (forward primer CAAGGCACAGACTAAGCAAGGAGAG (SEQ ID NO: 63); reverse primer GGCAAAATTTTCATAGGG-GAGAGAAATGGGTCATT (SEQ ID NO: 64)).

[0238] Expected results are shown in FIG. 31. Here, six of the 96 wells test positive for allele A, confirming the presence of cells of fetal origin, because the maternal genotype (BB) is known and cannot be positive for allele A. DNA from positive wells may be pooled for further analysis or analyzed individually.

Example 6

Quantitative Genotyping Using Molecular Inversion Probes for Trisomy Diagnosis on Fetal Cells

[0239] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. Quantitative genotyping can then be used to detect chromosome copy number changes. FIG. 5 depicts a flow chart depicting the major steps involved in detecting chromosome copy number changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[0240] Perform multiplex PCR and nested PCR: PCR primer pairs for multiple (40-100) highly polymorphic SNPs can then be added to each well in the microtiter plate. For example, SNPs primers can be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Multiple (~10) SNPs would be designed for each chromosome of interest to allow for non-informative genotypes and to ensure accurate results. The SNPs listed in the Table below can be used to performed analysis and associated PCR primers can be designed as described below.

[0241] PCR primers would be chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers would be designed to generate amplicons 70-100 bp in size to increase the performance of the multiplex PCR. The primers would contain a 22 bp tag on the 5' which is used in the genotyping analysis. Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 μl per well, Taq polymerase buffer (Perkin-Elmer), 200 μM dNTPs, primer, 1.5 mM MgCl2 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95° C. for 5 min, 41 cycles at 94, 60 and 72° C. for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60° C. depending on the primer pair being amplified. Final extension can be for 10 min.

[0242] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. Two ul aliquot of each PCR reaction is diluted 40 fold (to 80 ul total) with nuclease free water from the PCR kit. A no template or negative control is generated to test for contamination. The amplification with the nested PCR primers is run with an annealing temperature of 60° C.-68° C. depending on the primer pair being amplified.

Nested PCR cycle		
Step	Temp (C.)	Time (mins)
1.0	95	0.5
2.0	94	0.5
3.0	X	1.5
4.0	72	1.5
5.0	cycle to step 2, 44 times	
6.0	72	10

Master mix for nested primers		
	1 rxn	9 rxns
2X Q Mix	12.5	
	112.5	
titanium	0.5	4.5
Q	2.5	
	22.5	

SNPs that can be used for fetal cell analysis			
Chromosome 13	Chromosome 18	Chromosome 21	Chromosome X
refSNP rs9510053	refSNP rs584853	refSNP rs469000	refSNP rs6608727
refSNP rs7339372	refSNP rs2345588	refSNP rs7278903	refSNP rs2015487
refSNP rs9580269	refSNP rs9973072	refSNP rs1004044	refSNP rs5953330
refSNP rs724946	refSNP rs7504787	refSNP rs11910419	refSNP rs5953330
refSNP rs11842845	refSNP rs4303617	refSNP rs2832890	refSNP rs1984695
refSNP rs7490040	refSNP rs9947441	refSNP rs1785477	refSNP rs5906775
refSNP rs12430585	refSNP rs2912334	refSNP rs2250226	refSNP rs5951325
refSNP rs713280	refSNP rs11659665	refSNP rs2243594	refSNP rs11798710
refSNP rs202090	refSNP rs8098249	refSNP rs10483087	refSNP rs4898352
refSNP rs5000966	refSNP rs12968582	refSNP rs855262	refSNP rs5987079

-continued

Master mix for nested primers

	1 rxn	9 rxns
water	3.3	
	29.3	
5 uM primers	1.3	
40X diluted template	5.0	
	45.0	
	25.0	
	213.8	

[0243] Genotyping using MIP technology with bin specific tags: The Molecular Inversion Probe (MIP) technology developed by Affymetrix (Santa Clara, Calif.) can genotype 20,000 SNPs or more in a single reaction. In the typical MIP assay, each SNP would be assigned a 22 bp DNA tag which allows the SNP to be uniquely identified during the highly parallel genotyping assay. In this example, the DNA tags serve two roles: (1) determine the identity of the different SNPs and (2) determine the identity of the well from which the genotype was derived. For example, a total of 20,000 tags would be required to genotype the same 40 SNPs in 500 wells different wells (4 chromosomes×10 SNPs×500 wells)

[0244] The tagged MIP probes would be combined with the amplicons from the initial multiplex single-cell PCR (or nested PCR) and the genotyping reactions would be performed. The probe/template mix would be divided into 4 tubes each containing a different nucleotide (e.g. G, A, T or C). Following an extension and ligation step, the mixture would be treated with exonuclease to remove all linear molecules and the tags of the surviving circular molecules would be amplified using PCR. The amplified tags form all of the bins would then be pooled and hybridized to a single DNA microarray containing the complementary sequences to each of the 20,000 tags.

[0245] Identify bins with non-maternal alleles (e.g. fetal cells): The first step in the data analysis procedure would be to use the 22 bp tags to sort the 20,000 genotypes into bins which correspond to the individual wells of the original microtiter plates. The second step would be to identify bins contain non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins would provide an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles would be detected by 40 independent SNPs which provide an extremely high level of confidence in the result.

[0246] Detect ploidy for chromosomes 13, 18, and 21: After identifying approximately 10 bins that contain fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18, 21 and X by comparing ratio of maternal to paternal alleles for each of the 10 SNPs on each chromosome. The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the approximate 10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 7

Ultra-Deep Sequencing for Trisomy Diagnosis on Fetal Cells

[0247] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. The

enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[0248] Perform multiplex PCR and Ultra-Deep Sequencing with bin specific tags: PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) are then added to each well in the microtiter plate. The polymorphic STRs listed in the Table below can be used to performed analysis and associated PCR primers can be designed.

STR loci that can be used for fetal cell analysis

MARKER	CHROMOSOME LOCATION
D21S1414	21q21
MBP	18q23-ter
D13S634	13q14.3-22
D13S631	13q31-32
D18S535	18q12.2-12.3
D21S1412	21(S171-S198)
D21S1411	21q22.3
D21S11	21q21
D18S386	18q22.1-18q22.2
D13S258	13q21.2-13q31
D13S303	13q22-13q31
D18S1002	18q11

[0249] The primers for each STR will have two important features. First, each of the primers will contain a common ~18 bp sequence on the 5' end which is used for the subsequent DNA cloning and sequencing procedures. Second, each well in the microtiter plate is assigned a unique~6 bp DNA tag sequence which is incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR which makes it possible to analyze the amplicons in parallel more cost effectively during the ultra-deep sequencing procedure. DNA tags of length~6 by provide a compromise between information content (4096 potential bins) and the cost of synthesizing primers.

[0250] Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 μl per well, Taq polymerase buffer (Perkin-Elmer), 200 μM dNTPs, primer, 1.5 mM MgC12 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95° C. for 5 min, 41 cycles at 94, 60 and 72° C. for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60° C. depending on the primer pair being amplified. Final extension can be for 10 min.

[0251] Following PCR, the amplicons from each of the wells in the microtiter plate are pooled, purified and analyzed using a single-molecule sequencing strategy as described in Margulies et al. Nature 437(2005) 376-380. Briefly, the

amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA-carrying beads are isolated in separate 100 um aqueous droplets made through the creation of a PCR-reation-mix-ture-in-oil emulsion. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence, which all remain bound to the bead. Finally, the beads are placed into a highly parallel sequencing-by-synthesis machine which can generate over 400,000 sequence reads (~100 bp per read) in a single 4 hour run.

[0252] Ultra-deep sequencing provides an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs and the error rates of the multiplex PCR and the Poisson sampling statistics associated with the sequencing procedures. Statis-tical models which may account for variables in amplification can be used to detect ploidy changes with high levels of confidence. Using this statistical model it can be predicted that~100,000 to 300,000 sequence reads will be required to analyze each patient, with ~3 to 10 STR loci per chromosome. Specifically, ~33 reads for each of 12 STRs in each of the individual wells of the microtiter plate will be read (33 reads× 12 STRs per well×500 wells=200,000 reads).

[0253] Identify bins with non-maternal alleles (e.g. fetal cells): The first step in the data analysis procedure would be to use the 6 bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microti-ter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell frac-tion (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Deter-mining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell popu-lation. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[0254] Detect ploidy for chromosomes 13, 18, and 21: After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confi-

dence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 8

Sequencing for Trisomy Diagnosis on Fetal Cells

[0255] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1 and 2. Sequencing methods can then be used to detect chromosome copy number changes. FIG. 4 depicts a flow chart depicting the major steps involved in detecting chromosome copy num-ber changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 mater-nal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approxi-mately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[0256] Perform Multiplex PCR and Sequencing with bin Specific tags:

[0257] PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) can be added to each well in the microtiter plate. For example, STRs could be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Typically, four or more STRs should be analyzed per chromosome of interest to ensure accurate detection of aneuploidy.

[0258] The primers for each STR can be designed with two important features. First, each primer can contain a common ~18 bp sequence on the 5' end which can be used for the subsequent DNA cloning and sequencing procedures. Sec-ond, each well in the microtiter plate can be assigned a unique ~6 bp DNA tag sequence which can be incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR, which makes pos-sible to analyze the amplicons in parallel during the ultra-deep sequencing procedure. Furthermore, nested PCR strat-egies for the STR amplification can achieve higher reliability of amplification from single cells.

[0259] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[0260] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the tar-get nucleic acid sequence. Sequence-by-synthesis can be ini-tiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immedi-ately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incor-poration of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Pub-

lication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100, 000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[0261] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase, apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next, deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

[0262] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins.

[0263] Identify bins with non-maternal alleles (e.g. fetal cells): The first step in the data analysis procedure would be to use the 6 bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microtiter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell fraction (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell population. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[0264] Detect ploidy for chromosomes 13, 18, and 21: After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic diallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confidence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 9

Device Embodiment

[0265] Microfluidic devices of the invention were designed by computer-aided design (CAD) and microfabricated by photolithography. A two-step process was developed in which a blood sample is first debulked to remove the large population of small cells, and then the rare target epithelial cells target cells are recovered by immunoaffinity capture. The devices were defined by photolithography and etched into a silicon substrate based on the CAD-generated design. The cell enrichment module, which is approximately the size of a standard microscope slide, contains 14 parallel sample processing sections and associated sample handling channels that connect to common sample and buffer inlets and product and waste outlets. Each section contains an array of microfabricated obstacles that is optimized to enrich the target cell type by hydrodynamic size via displacement of the larger cells into the product stream. In this example, the microchip was designed to separate red blood cells (RBCs) and platelets from the larger leukocytes and CTCs. Enriched populations of target cells were recovered from whole blood passed through the device. Performance of the cell enrichment microchip was evaluated by separating RBCs and platelets from white blood cells (WBCs) in normal whole blood (FIG. 15). In cancer patients, CTCs are found in the larger WBC fraction. Blood was minimally diluted (30%), and a 6 ml sample was processed at a flow rate of up to 6 ml/hr. The product and waste stream were evaluated in a Coulter Model "A^C-T diff" clinical blood analyzer, which automatically distinguishes, sizes, and counts different blood cell populations. The enrichment chip achieved separation of RBCs from WBCs, in which the WBC fraction had >99% retention of nucleated cells, >99% depletion of RBCs, and >97% depletion of platelets. Representative histograms of these cell fractions are shown in FIG. 6. Routine cytology confirmed the high degree of enrichment of the WBC and RBC fractions (FIG. 17).

[0266] Next, epithelial cells were recovered by affinity capture in a microfluidic module that is functionalized with immobilized antibody. A capture module with a single chamber containing a regular array of antibody-coated microfabricated obstacles was designed. These obstacles are disposed to maximize cell capture by increasing the capture area approximately four-fold, and by slowing the flow of cells under laminar flow adjacent to the obstacles to increase the contact time between the cells and the immobilized antibody.

The capture modules may be operated under conditions of relatively high flow rate but low shear to protect cells against damage. The surface of the capture module was functionalized by sequential treatment with 10% silane, 0.5% gluteraldehyde, and avidin, followed by biotinylated anti-EpCAM. Active sites were blocked with 3% bovine serum albumin in PBS, quenched with dilute Tris HCl, and stabilized with dilute L-histidine. Modules were washed in PBS after each stage and finally dried and stored at room temperature. Capture performance was measured with the human advanced lung cancer cell line NCI-H 1650 (ATCC Number CRL-5883). This cell line has a heterozygous 15 by in-frame deletion in exon 19 of EGFR that renders it susceptible to gefitinib. Cells from confluent cultures were harvested with trypsin, stained with the vital dye Cell Tracker Orange (CMRA reagent, Molecular Probes, Eugene, Oreg.), resuspended in fresh whole blood, and fractionated in the microfluidic chip at various flow rates. In these initial feasibility experiments, cell suspensions were processed directly in the capture modules without prior fractionation in the cell enrichment module to debulk the red blood cells; hence, the sample stream contained normal blood red cells and leukocytes as well as tumor cells. After the cells were processed in the capture module, the device was washed with buffer at a higher flow rate (3 ml/hr) to remove the nonspecifically bound cells. The adhesive top was removed and the adherent cells were fixed on the chip with paraformaldehyde and observed by fluorescence microscopy. Cell recovery was calculated from hemacytometer counts; representative capture results are shown in Table 4. Initial yields in reconstitution studies with unfractionated blood were greater than 60% with less than 5% of non-specific binding.

TABLE 4

Run number	Avg. flow rate	Length of run	No. cells processed	No. cells captured	Yield
1	3.0	1 hr	150,000	38,012	25%
2	1.5	2 hr	150,000	30,000/ml	60%
3	1.08	2 hr	108,000	68,661	64%
4	1.21	2 hr	121,000	75,491	62%

[0267] Next, NCI-H 1650 cells that were spiked into whole blood and recovered by size fractionation and affinity capture as described above were successfully analyzed in situ. In a trial run to distinguish epithelial cells from leukocytes, 0.5 ml of a stock solution of fluorescein-labeled CD45 pan-leukocyte monoclonal antibody were passed into the capture module and incubated at room temperature for 30 minutes. The module was washed with buffer to remove unbound antibody, and the cells were fixed on the chip with 1% paraformaldehyde and observed by fluorescence microscopy. As shown in FIG. 18, the epithelial cells were bound to the obstacles and floor of the capture module. Background staining of the flow passages with CD45 pan-leukocyte antibody is visible, as are several stained leukocytes, apparently because of a low level of non-specific capture.

Example 10

Device Embodiments

[0268] A design for preferred device embodiments of the invention is shown in FIG. 19A, and parameters corresponding to three preferred device embodiments associated with this design are shown in FIGS. 19B and 19C. These embodiments are particularly useful for enrich epithelial cells from blood.

Example 11

Determining Counts for Large Cell Types

[0269] Using the methods of the invention, a diagnosis of the absence, presence, or progression of cancer may be based on the number of cells in a cellular sample that are larger than a particular cutoff size. For example, cells with a hydrodynamic size of 14 microns or larger may be selected. This cutoff size would eliminate most leukocytes. The nature of these cells may then be determined by downstream molecular or cytological analysis.

[0270] Cell types other than epithelial cells that would be useful to analyze include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts indicative of a disease state. Furthermore, determining separate counts for epithelial cells, e.g., cancer cells, and other cell types, e.g., endothelial cells, followed by a determination of the ratios between the number of epithelial cells and the number of other cell types, may provide useful diagnostic information.

[0271] A device of the invention may be configured to isolate targeted subpopulations of cells such as those described above, as shown in FIGS. 20A-D. A size cutoff may be selected such that most native blood cells, including red blood cells, white blood cells, and platelets, flow to waste, while non-native cells, which could include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts, are collected in an enriched sample. This enriched sample may be further analyzed.

[0272] Using a device of the invention, therefore, it is possible to isolate a subpopulation of cells from blood or other bodily fluids based on size, which conveniently allows for the elimination of a large proportion of native blood cells when large cell types are targeted. As shown schematically in FIG. 21, a device of the invention may include counting means to determine the number of cells in the enriched sample, or the number of cells of a particular type, e.g., cancer cells, within the enriched sample, and further analysis of the cells in the enriched sample may provide additional information that is useful for diagnostic or other purposes.

Example 12

Method for Detection of EGFR Mutations

[0273] A blood sample from a cancer patient is processed and analyzed using the devices and methods of the invention, resulting in an enriched sample of epithelial cells containing CTCs. This sample is then analyzed to identify potential EGFR mutations. The method permits both identification of known, clinically relevant EGFR mutations as well as discovery of novel mutations. An overview of this process is shown in FIG. 22.

[0274] Below is an outline of the strategy for detection and confirmation of EGFR mutations:

[0275] 1) Sequence CTC EGFR mRNA

[0276] a) Purify CTCs from blood sample;

[0277] b) Purify total RNA from CTCs;

[0278] c) Convert RNA to cDNA using reverse transcriptase;

[0279] d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates; and

[0280] e) Purify the nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21.

[0281] 2) Confirm RNA sequence using CTC genomic DNA

[0282] a) Purify CTCs from blood sample;

[0283] b) Purify genomic DNA (gDNA) from CTCs;

[0284] c) Ample exons 18, 19, 20, and/or 21 via PCR reactions; and

[0285] d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing.

[0286] Further details for each step outlined above are as follows.

[0287] 1) Sequence CTC EGFR mRNA

[0288] a) Purify CTCs from blood sample. CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

[0289] b) Purify total RNA from CTCs. Total RNA is then purified from isolated CTC populations using, e.g., the Qiagen Micro RNeasy kit, or a similar total RNA purification protocol from another manufacturer; alternatively, standard RNA purification protocols such as guanidium isothiocyanate homogenization followed by phenol/chloroform extraction and ethanol precipitation may be used. One such method is described in "Molecular Cloning—A Laboratory Manual, Second Edition" (1989) by J. Sambrook, E. F. Fritch and T. Maniatis, p. 7.24.

[0290] c) Convert RNA to cDNA using reverse transcriptase. cDNA reactions are carried out based on the protocols of the supplier of reverse transcriptase. Typically, the amount of input RNA into the cDNA reactions is in the range of 10 picograms (pg) to 2 micrograms (μg) total RNA. First-strand DNA synthesis is carried out by hybridizing random 7 mer DNA primers, or oligo-dT primers, or gene-specific primers, to RNA templates at 65° C. followed by snap-chilling on ice. cDNA synthesis is initiated by the addition of iScript Reverse Transcriptase (BioRad) or SuperScript Reverse Transcriptase (Invitrogen) or a reverse transcriptase from another commercial vendor along with the appropriate enzyme reaction buffer. For iScript, reverse transcriptase reactions are carried out at 42° C. for 30-45

minutes, followed by enzyme inactivation for 5 minutes at 85° C. cDNA is stored at –20° C. until use or used immediately in PCR reactions. Typically, cDNA reactions are carried out in a final volume of 20 μl, and 10% (2 μl) of the resultant cDNA is used in subsequent PCR reactions.

[0291] d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates. cDNA from the reverse transcriptase reactions is mixed with DNA primers specific for the region of interest (FIG. **23**). See Table 5 for sets of primers that may be used for amplification of exons 18-21. In Table 5, primer set M13(+)/M12(–) is internal to primer set M11(+)/M14(–). Thus primers M13(+) and M12(–) may be used in the nested round of amplification, if primers M11(+) and M14(–) were used in the first round of expansion. Similarly, primer set M11(+)/M14(–) is internal to primer set M15(+)/M16(–), and primer set M23(+)/M24 (–) is internal to primer set M21(+)/M22(–). Hot Start PCR reactions are performed using Qiagen Hot-Star Taq Polymerase kit, or Applied Biosystems HotStart Taq-Man polymerase, or other Hot Start thermostable polymerase, or without a hot start using Promega GoTaq Green Taq Polymerase master mix, TaqMan DNA polymerase, or other thermostable DNA polymerase. Typically, reaction volumes are 50 μl, nucleotide triphosphates are present at a final concentration of 200 μM for each nucleotide, $MgCl_2$ is present at a final concentration of 1-4 mM, and oligo primers are at a final concentration of 0.5 μM. Hot start protocols begin with a 10-15 minute incubation at 95° C., followed by 40 cycles of 94° C. for one minute (denaturation), 52° C. for one minute (annealing), and 72° C. for one minute (extension). A 10 minute terminal extension at 72° C. is performed before samples are stored at 4° C. until they are either used as template in the second (nested) round of PCRs, or purified using QiaQuick Spin Columns (Qiagen) prior to sequencing. If a hot-start protocol is not used, the initial incubation at 95° C. is omitted. If a PCR product is to be used in a second round of PCRs, 2 μl (4%) of the initial PCR product is used as template in the second round reactions, and the identical reagent concentrations and cycling parameters are used.

TABLE 5

Primer Sets for expanding EGFR mRNA around Exons 18-21

Name		SEQ ID NO	Sequence (5' to 3')	cDNA Coordinates		Amplicon Size
NXK-M11	(+)	1	TTGCTGCTGGTGGTGGC	(+)	1966-1982	813
NXK-M14	(–)	2	CAGGGATTCCGTCATATGGC	(–)	2778-2759	
NXK-M13	(+)	3	GATCGGCCTCTTCATGCG	(+)	1989-2006	747
NXK M12	(–)	4	GATCCAAAGGTCATCAACTCCC	(–)	2735-2714	
NXK-M15	(+)	5	GCTGTCCAACGAATGGGC	(+)	1904-1921	894
NXK-M16	(–)	6	GGCGTTCTCCTTTCTCCAGG	(–)	2797-2778	
NXK-M21	(+)	7	ATGCACTGGGCCAGGTCTT	(+)	1881-1899	944
NXK-M22	(–)	8	CGATGGTACATATGGGTGGCT	(–)	2824-2804	
NXK-M23	(+)	9	AGGCTGTCCAACGAATGGG	(+)	1902-1920	904
NXK-M24	(–)	10	CTGAGGGAGGCGTTCTCCT	(–)	2805-2787	

[0292] e) Purify the nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21. Sequencing is performed by ABI automated fluorescent sequencing machines and fluorescence-labeled DNA sequencing ladders generated via Sanger-style sequencing reactions using fluorescent dideoxynucleotide mixtures. PCR products are purified using Qiagen Quick-Spin columns, the Agencourt AMPure PCR Purification System, or PCR product purification kits obtained from other vendors. After PCR products are purified, the nucleotide concentration and purity is determined with a Nanodrop 7000 spectrophotometer, and the PCR product concentration is brought to a concentration of 25 ng/µl. As a quality control measure, only PCR products that have a UV-light absorbance ratio (A_{260}/A_{280}) greater than 1.8 are used for sequencing. Sequencing primers are brought to a concentration of 3.2 pmol/µl.

[0293] 2) Confirm RNA Sequence Using CTC Genomic DNA

[0294] a) Purify CTCs from blood sample. As above, CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

[0295] b) Purify genomic DNA (gDNA) from CTCs. Genomic DNA is purified using the Qiagen DNeasy Mini kit, the Invitrogen ChargeSwitch gDNA kit, or another commercial kit, or via the following protocol:

[0296] 1. Cell pellets are either lysed fresh or stored at −80° C. and are thawed immediately before lysis.

[0297] 2. Add 500 µl 50 mM Tris pH 7.9/100 mM EDTA/0.5% SDS (TES buffer).

[0298] 3. Add 12.5 µl Proteinase K (IBI15406, 20 mg/ml), generating a final [ProtK]=0.5 mg/ml.

[0299] 4. Incubate at 55° C. overnight in rotating incubator.

[0300] 5. Add 20 µl of RNase cocktail (500 U/ml RNase A+20,000 U/ml RNase T1, Ambion #2288) and incubate four hours at 37° C.

[0301] 6. Extract with Phenol (Kodak, Tris pH 8 equilibrated), shake to mix, spin 5 min. in tabletop centrifuge.

[0302] 7. Transfer aqueous phase to fresh tube.

[0303] 8. Extract with Phenol/Chloroform/Isoamyl alcohol (EMD, 25:24:1 ratio, Tris pH 8 equilibrated), shake to mix, spin five minutes in tabletop centrifuge.

[0304] 9. Add 50 µl 3M NaOAc pH=6.

[0305] 10. Add 500 pl EtOH.

[0306] 11. Shake to mix. Strings of precipitated DNA may be visible. If anticipated DNA concentration is very low, add carrier nucleotide (usually yeast tRNA).

[0307] 12. Spin one minute at max speed in tabletop centrifuge.

[0308] 13. Remove supernatant.

[0309] 14. Add 500 µl 70% EtOH, Room Temperature (RT)

[0310] 15. Shake to mix.

[0311] 16. Spin one minute at max speed in tabletop centrifuge.

[0312] 17. Air dry 10-20 minutes before adding TE.

[0313] 18. Resuspend in 400 µl TE. Incubate at 65° C. for 10 minutes, then leave at RT overnight before quantitation on Nanodrop.

[0314] c) Amplify exons 18, 19, 20, and/or 21 via PCR reactions. Hot start nested PCR amplification is carried out as described above in step 1d. except that there is no nested round of amplification. The initial PCR step may be stopped during the log phase in order to minimize possible loss of allele-specific information during amplification. The primer sets used for expansion of EGFR exons 18-21 are listed in Table 6 (see also Paez et al., Science 304:1497-1500 (Supplementary Material) (2004)).

TABLE 6

Primer sets for expanding EGFR genomic DNA

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1 (+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2 (−)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3 (+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4 (−)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1 (+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2 (−)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3 (+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4 (−)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1 (+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2 (−)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3 (+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4 (−)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1 (+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2 (−)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3 (+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4 (−)	26	CATCCTCCCCTGCATGTGT	21	

28

[0315] d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing. An aliquot of the PCR amplicons is used as template in a multiplexed allele-specific quantitative PCR reaction using TaqMan PCR 5' Nuclease assays with an Applied Biosystems model 7500 Real Time PCR machine (FIG. 24). This round of PCR amplifies subregions of the initial PCR product specific to each mutation of interest. Given the very high sensitivity of Real Time PCR, it is possible to obtain complete information on the mutation status of the EGFR gene even if as few as 10 CTCs are isolated. Real Time PCR provides quantification of allelic sequences over 8 logs of input DNA concentrations; thus, even heterozygous mutations in impure populations are easily detected using this method.

[0316] Probe and primer sets are designed for all known mutations that affect gefitinib responsiveness in NSCLC patients, including over 40 such somatic mutations, including point mutations, deletions, and insertions, that have been reported in the medical literature. For illustrative purposes, examples of primer and probe sets for five of the point mutations are listed in Table 7. In general, oligonucleotides may be designed using the primer optimization software program Primer Express (Applied Biosystems), with hybridization conditions optimized to distinguish the wild type EGFR DNA sequence from mutant alleles. EGFR genomic DNA amplified from lung cancer cell lines that are known to carry EGFR mutations, such as H358 (wild type), H1650 (15-bp deletion, Δ2235-2249), and H1975 (two point mutations, 2369 C→T, 2573 T→G), is used to optimize the allele-specific Real Time

PCR reactions. Using the TaqMan 5' nuclease assay, allele-specific labeled probes specific for wild type sequence or for known EGFR mutations are developed. The oligonucleotides are designed to have melting temperatures that easily distinguish a match from a mismatch, and the Real Time PCR conditions are optimized to distinguish wild type and mutant alleles. All Real Time PCR reactions are carried out in triplicate.

[0317] Initially, labeled probes containing wild type sequence are multiplexed in the same reaction with a single mutant probe. Expressing the results as a ratio of one mutant allele sequence versus wild type sequence may identify samples containing or lacking a given mutation. After conditions are optimized for a given probe set, it is then possible to multiplex probes for all of the mutant alleles within a given exon within the same Real Time PCR assay, increasing the ease of use of this analytical tool in clinical settings.

[0318] A unique probe is designed for each wild type allele and mutant allele sequence. Wild-type sequences are marked with the fluorescent dye VIC at the 5' end, and mutant sequences with the fluorophore FAM. A fluorescence quencher and Minor Groove Binding moiety are attached to the 3' ends of the probes. ROX is used as a passive reference dye for normalization purposes. A standard curve is generated for wild type sequences and is used for relative quantitation. Precise quantitation of mutant signal is not required, as the input cell population is of unknown, and varying, purity. The assay is set up as described by ABI product literature, and the presence of a mutation is confirmed when the signal from a mutant allele probe rises above the background level of fluorescence (FIG. 25), and this threshold cycle gives the relative frequency of the mutant allele in the input sample.

TABLE 7

Probes and Primers for Allele-Specific qPCR

Name	SEQ ID NO	Sequence (5' to 3', mutated position in bold)	EMBL Chromosome 7 Genomic Coordinates		Description	Mutation
NXK-M01	27	CCGCAGCATGTCAAGATCAC	(+)	55,033,694-55,033,713	(+) primer	L858R
NXK-M02	28	TCCTTCTGCATGGTATTCTTTCTCT	(−)	55,033,769-55,033,745	(−) primer	
Pwt-L858R	29	VIC-TTTGGGCTGGCCAA-MGB	(+)	55,033,699-55,033,712	WT allele probe	
Pmut-L858R	30	FAM-TTTTGGGCGGGCCA-MGB	(+)	55,033,698-55,033,711	Mutant allele probe	
NXK-M03	31	ATGGCCAGCGTGGACAA	(+)	55,023,207-55,023,224	(+) primer	T790M
NXK-M04	32	AGCAGGTACTGGGAGCCAATATT	(−)	55,023,355-55,023,333	(−) primer	
Pwt-T790M	33	VIC-ATGAGCTGCGTGATGA-MGB	(−)	55,023,290-55,023,275	WT allele probe	
Pmut-T790M	34	FAM-ATGAGCTGCATGATGA-MGB	(−)	55,023,290-55,023,275	Mutant allele probe	
NXK-M05	35	GCCTCTTACACCCAGTGGAGAA	(+)	55,015,831-55,015,852	(+) primer	G719S, C
NXK-ex18.5	36	GCCTGTGCCAGGGACCTT	(−)	55,015,965-55,015,948	(−) primer	
Pwt-G719SC	37	VIC-ACCGGAGCCCAGCA-MGB	(−)	55,015,924-55,015,911	WT allele probe	
Pmut-G719S	38	FAM-ACCGGAGCTCAGCA-MGB	(−)	55,015,924-55,015,911	Mutant allele probe	
mut-G719C	39	FAM-ACCGGAGCACAGCA-MGB	(−)	55,015,924-55,015,911	Mutant allele probe	

TABLE 7-continued

Probes and Primers for Allele-Specific qPCR

Name	SEQ ID NO	Sequence (5' to 3', mutated position in bold)	EMBL Chromosome 7 Genomic Coordinates	Description	Mutation
NXK-ex21.5	40	ACAGCAGGGTCTTCTCTGTTTCAG	(+) 55,033,597-55,033,620	(+) primer	H835L
NXK-M10	41	ATCTTGACATGCTGCGGTGTT	(-) 55,033,710-55,033,690	(-) primer	
Pwt-H835L	42	VIC-TTGGTGCACCGCGA-MGB	(+) 55,033,803-55,033,816	WT allele probe	
Pmut-H835L	43	FAM-TGGTGCTCCGCGAC-MGB	(+) 55,033,803-55,033,816	Mutant allele probe	
NXK-M07	52	TGGATCCCAGAAGGTGAGAAA	(+) 55,016,630-55,016,650	(+) primer	delE746-A750
NXK-ex19.5	53	AGCAGAAACTCACATCGAGGATTT	(-) 55,016,735-55,016,712	(-) primer	
Pwt-delE746-A750	54	AAGGAATTAAGAGAAGCAA	(+) 55,016,681-55,016,699	WT allele probe	
Pmut-delE746-A750var1	55	CTATCAAAACATCTCC	(+) 55,016,676-55,016,691	Mutant allele probe, variant 1	
Pmut-delE746-A750var1	56	CTATCAAGACATCTCC	(+) 55,016,676-55,016,691	Mutant allele probe, variant 2	

Example 13

Absence of EGFR Expression in Leukocytes

[0319]   To test whether EGFR mRNA is present in leuko-cytes, several PCR experiments were performed. Four sets of primers, shown in Table 8, were designed to amplify four corresponding genes:

[0320]   1) BCKDK (branched-chain a-ketoacid dehydro-genase complex kinase)—a "housekeeping" gene expressed in all types of cells, a positive control for both leukocytes and tumor cells;

[0321]   2) CD45—specifically expressed in leukocytes, a positive control for leukocytes and a negative control for tumor cells;

[0322]   3) EpCaM—specifically expressed in epithelial cells, a negative control for leukocytes and a positive control for tumor cells; and

[0323]   4) EGFR—the target mRNA to be examined.

[0324]   Total RNAs of approximately 9×10⁶ leukocytes iso-lated using a cell enrichment device of the invention (cutoff size 4 μm) and 5×10⁶ H1650 cells were isolated by using RNeasy mini kit (Qiagen). Two micrograms of total RNAs from leukocytes and H1650 cells were reverse transcribed to obtain first strand cDNAs using 100 pmol random hexamer (Roche) and 200 U Superscript II (Invitrogen) in a 20 μl reaction. The subsequent PCR was carried out using 0.5 μl of the first strand cDNA reaction and 10 pmol of forward and reverse primers in total 25 μl of mixture. The PCR was run for 40 cycles of 95° C. for 20 seconds, 56° C. for 20 seconds, and 70° C. for 30 seconds. The amplified products were separated on a 1% agarose gel. As shown in FIG. 26A, BCKDK was found to be expressed in both leukocytes and H1650 cells; CD45 was expressed only in leukocytes; and both EpCAM and EGFR were expressed only in H1650 cells. These results, which are fully consistent with the profile of EGFR expres-sion shown in FIG. 26B, confirmed that EGFR is a particu-

TABLE 8

Name	SEQ ID NO	Sequence (5' to 3')	Description	Amplicon Size
BCKD_1	44	AGTCAGGACCCATGCACGG	BCKDK (+) primer	273
BCKD_2	45	ACCCAAGATGCAGCAGTGTG	BCKDK (-) primer	
CD45_1	46	GATGTCCTCCTTGTTCTACTC	CD45 (+) primer	263
CD45_2	47	TACAGGGAATAATCGAGCATGC	CD45 (-) primer	
EpCAM_1	48	GAAGGGAAATAGCAAATGGACA	EpCAM (+) primer	222
EpCAM_2	49	CGATGGAGTCCAAGTTCTGG	EpCAM (-) primer	
EGFR_1	50	AGCACTTACAGCTCTGGCCA	EGFR (+) primer	371
EGFR_2	51	GACTGAACATAACTGTAGGCTG	EGFR (-) primer	

larly useful target for assaying mixtures of cells that include both leukocytes and cancer cells, because only the cancer cells will be expected to produce a signal.

Example 14

EGFR Assay with low Quantities of Target RNA or High Quantities of Background RNA

[0325] In order to determine the sensitivity of the assay described in Example 12, various quantities of input NSCLC cell line total RNA were tested, ranging from 100 pg to 50 ng. The results of the first and second EGFR PCR reactions (step 1d, Example 12) are shown in FIG. 27. The first PCR reaction was shown to be sufficiently sensitive to detect 1 ng of input RNA, while the second round increased the sensitivity to 100 pg or less of input RNA. This corresponds to 7-10 cells, demonstrating that even extremely dilute samples may generate detectable signals using this assay.

[0326] Next, samples containing 1 ng of NCI-H1975 RNA were mixed with varying quantities of peripheral blood mononuclear cell (PBMC) RNA ranging from 1 ng to 1 μg and used in PCR reactions as before. As shown in FIG. 28A, the first set of PCR reactions demonstrated that, while amplification occurred in all cases, spurious bands appeared at the highest contamination level. However, as shown in FIG. 28B, after the second, nested set of PCR reactions, the desired specific amplicon was produced without spurious bands even at the highest contamination level. Therefore, this example demonstrates that the EGFR PCR assays described herein are effective even when the target RNA occupies a tiny fraction of the total RNA in the sample being tested.

[0327] Table 8 lists the RNA yield in a variety of cells and shows that the yield per cell is widely variable, depending on the cell type. This information is useful in order to estimate the amount of target and background RNA in a sample based on cell counts. For example, 1 ng of NCL-H1975 RNA corresponds to approximately 100 cells, while 1 μg of PBMC RNA corresponds to approximately 10^6 cells. Thus, the highest contamination level in the above-described experiment, 1,000:1 of PBMC RNA to NCL-H1975 RNA, actually corresponds to a 10,000:1 ratio of PBMCs to NCL-H1975 cells. Thus, these data indicate that EGFR may be sequenced from as few as 100 CTCs contaminated by as many as 10^6 leukocytes.

TABLE 8

	RNA Yield versus Cell Type		
Cells	Count	RNA Yield	[RNA]/Cell
NCI-H1975	2×10^6	26.9 μg	13.5 pg
NCI-H1650	2×10^6	26.1 μg	13.0 pg
H358	2×10^6	26.0 μg	13.0 pg
HT29	2×10^6	21.4 μg	10.7 pg
MCF7	2×10^6	25.4 μg	12.7 pg
PBMC #1	19×10^6	10.2 μg	0.5 pg
PBMC #2	16.5×10^6	18.4 μg	1.1 pg

[0328] Next, whole blood spiked with 1,000 cells/ml of Cell Tracker (Invitrogen)-labeled H1650 cells was run through the capture module chip of FIG. 19C. To avoid inefficiency in RNA extraction from fixed samples, the captured H1650 cells were immediately counted after running and subsequently lysed for RNA extraction without formaldehyde fixation. Approximately 800 captured H1650 cells and >10,000 contaminated leukocytes were lysed on the chip with 0.5 ml of 4M guanidine thiocyanate solution. The lysate was extracted with 0.5 ml of phenol/chloroform and precipitated with 1 ml of ethanol in the presence of 10 mg of yeast tRNA as carrier. The precipitated RNAs were DNase 1-treated for 30 minutes and then extracted with phenol/chloroform and precipitated with ethanol prior to first strand cDNA synthesis and subsequent PCR amplification. These steps were repeated with a second blood sample and a second chip. The cDNA synthesized from chip 1 and chip 2 RNAs along with H1650 and leukocyte cDNAs were PCR amplified using two sets of primers, CD45_1 and CD45_2 (Table 7) as well as EGFR_5 (forward primer, 5'-GTTCGGCACGGTG-TATAAGG-3') (SEQ ID NO: 65[[_]]) and EGFR_6 (reverse primer, 5'-CTGGCCATCACGTAGGCTTC-3') (SEQ ID NO: 66[[_]]). EGFR_5 and EGFR_6 produce a 138 bp wild type amplified fragment and a 123 bp mutant amplified fragment in H1650 cells. The PCR products were separated on a 2.5% agarose gel. As shown in FIG. 29, EGFR wild type and mutant amplified fragments were readily detected, despite the high leukocyte background, demonstrating that the EGFR assay is robust and does not require a highly purified sample.

```
                         SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 70

<210> SEQ ID NO 1
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 1

ttgctgctgg tggtggc                                                   17


<210> SEQ ID NO 2
<211> LENGTH: 20
```

-continued

```
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 2

cagggattcc gtcatatggc                                              20


<210> SEQ ID NO 3
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 3

gatcggcctc ttcatgcg                                                18


<210> SEQ ID NO 4
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 4

gatccaaagg tcatcaactc cc                                           22


<210> SEQ ID NO 5
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 5

gctgtccaac gaatgggc                                                18


<210> SEQ ID NO 6
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 6

ggcgttctcc tttctccagg                                              20


<210> SEQ ID NO 7
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 7

atgcactggg ccaggtctt                                               19
```

-continued

```
<210> SEQ ID NO 8
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 8

cgatggtaca tatgggtggc t                                              21


<210> SEQ ID NO 9
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 9

aggctgtcca acgaatggg                                                 19


<210> SEQ ID NO 10
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 10

ctgagggagg cgttctcct                                                 19


<210> SEQ ID NO 11
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 11

tcagagcctg tgtttctacc aa                                             22


<210> SEQ ID NO 12
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 12

tggtctcaca ggaccactga tt                                             22


<210> SEQ ID NO 13
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 13

tccaaatgag ctggcaagtg                                                20
```

-continued

```
<210> SEQ ID NO 14
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 14

tcccaaacac tcagtgaaac aaa                                           23


<210> SEQ ID NO 15
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 15

aaataatcag tgtgattcgt ggag                                          24


<210> SEQ ID NO 16
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 16

gaggccagtg ctgtctctaa gg                                            22


<210> SEQ ID NO 17
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 17

gtgcatcgct ggtaacatcc                                               20


<210> SEQ ID NO 18
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 18

tgtggagatg agcagggtct                                               20


<210> SEQ ID NO 19
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 19
```

-continued

acttcacagc cctgcgtaaa c   21

<210> SEQ ID NO 20
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 20

atgggacagg cactgatttg t   21

<210> SEQ ID NO 21
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 21

atcgcattca tgcgtcttca   20

<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 22

atccccatgg caaactcttg   20

<210> SEQ ID NO 23
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 23

gcagcgggtt acatcttctt tc   22

<210> SEQ ID NO 24
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 24

cagctctggc tcacactacc ag   22

<210> SEQ ID NO 25
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

-continued

```
<400> SEQUENCE: 25

gcagcgggtt acatcttctt tc                                         22


<210> SEQ ID NO 26
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 26

catcctcccc tgcatgtgt                                             19


<210> SEQ ID NO 27
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 27

ccgcagcatg tcaagatcac                                            20


<210> SEQ ID NO 28
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 28

tccttctgca tggtattctt tctct                                      25


<210> SEQ ID NO 29
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 29

tttgggctgg ccaa                                                  14


<210> SEQ ID NO 30
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 30

ttttgggcgg gcca                                                  14


<210> SEQ ID NO 31
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
```

-continued

```
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 31

atggccagcg tggacaa                                                  17


<210> SEQ ID NO 32
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 32

agcaggtact gggagccaat att                                           23


<210> SEQ ID NO 33
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 33

atgagctgcg tgatga                                                   16


<210> SEQ ID NO 34
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 34

atgagctgca tgatga                                                   16


<210> SEQ ID NO 35
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 35

gcctcttaca cccagtggag aa                                            22


<210> SEQ ID NO 36
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 36

gcctgtgcca gggacctt                                                 18


<210> SEQ ID NO 37
<211> LENGTH: 14
<212> TYPE: DNA
```

-continued

```
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 37

accggagccc agca                                                         14


<210> SEQ ID NO 38
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 38

accggagctc agca                                                         14


<210> SEQ ID NO 39
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 39

accggagcac agca                                                         14


<210> SEQ ID NO 40
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 40

acagcagggt cttctctgtt tcag                                              24


<210> SEQ ID NO 41
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 41

atcttgacat gctgcggtgt t                                                 21


<210> SEQ ID NO 42
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 42

ttggtgcacc gcga                                                         14


<210> SEQ ID NO 43
```

-continued

```
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 43

tggtgctccg cgac                                                        14


<210> SEQ ID NO 44
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 44

agtcaggacc catgcacgg                                                   19


<210> SEQ ID NO 45
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 45

acccaagatg cagcagtgtg                                                  20


<210> SEQ ID NO 46
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 46

gatgtcctcc ttgttctact c                                                21


<210> SEQ ID NO 47
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 47

tacagggaat aatcgagcat gc                                               22


<210> SEQ ID NO 48
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 48

gaagggaaat agcaaatgga ca                                               22
```

-continued

```
<210> SEQ ID NO 49
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 49

cgatggagtc caagttctgg                                              20


<210> SEQ ID NO 50
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 50

agcacttaca gctctggcca                                              20


<210> SEQ ID NO 51
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 51

gactgaacat aactgtaggc tg                                           22


<210> SEQ ID NO 52
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 52

tggatcccag aaggtgagaa a                                            21


<210> SEQ ID NO 53
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 53

agcagaaact cacatcgagg attt                                         24


<210> SEQ ID NO 54
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 54
```

-continued

aaggaattaa gagaagcaa   19

<210> SEQ ID NO 55
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 55

ctatcaaaac atctcc   16

<210> SEQ ID NO 56
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 56

ctatcaagac atctcc   16

<210> SEQ ID NO 57
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 57

tcgagtgcat tccattccg   19

<210> SEQ ID NO 58
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 58

atggaatggc atcaaacgga a   21

<210> SEQ ID NO 59
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 59

tggctgtcca ttcca   15

<210> SEQ ID NO 60
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      oligonucleotide

-continued

```
<400> SEQUENCE: 60

atgcagcaag gcacagacta arcaaggaga sgcaaaattt tcrtagggga gagaaatggg      60

tcatt                                                                 65


<210> SEQ ID NO 61
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 61

atgcagcaag gcacagacta cg                                              22


<210> SEQ ID NO 62
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 62

agagggggaga gaaatgggtc att                                            23


<210> SEQ ID NO 63
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 63

caaggcacag actaagcaag gagag                                           25


<210> SEQ ID NO 64
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 64

ggcaaaattt tcatagggga gagaaatggg tcatt                                35


<210> SEQ ID NO 65
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 65

gttcggcacg gtgtataagg                                                 20


<210> SEQ ID NO 66
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
```

-continued

```
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      primer

<400> SEQUENCE: 66

ctggccatca cgtaggcttc                                                  20


<210> SEQ ID NO 67
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 67

cggagatggc cca                                                         13


<210> SEQ ID NO 68
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 68

gcaactcatc atgca                                                       15


<210> SEQ ID NO 69
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 69

ttttgggcgg gccaa                                                       15


<210> SEQ ID NO 70
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      probe

<400> SEQUENCE: 70

gaccgtttgg gagttgata                                                   19
```

1-54. (canceled)

55. A method for performing prenatal diagnosis of a fetal chromosomal aneuploidy in a biological sample obtained from a pregnant female subject, wherein the biological sample includes nucleic acid molecules, the method comprising: receiving the biological sample; sequencing at least a portion of a plurality of the nucleic acid molecules contained in the biological sample, wherein the sequenced portion represents a fraction of the human genome; based on the sequencing: determining a first amount of a first chromosome from sequences identified as originating from the first chromosome; determining a second amount of one or more second chromosomes from sequences identified as originating from one of the second chromosomes; determining a parameter from the first amount and the second amount; comparing the parameter to one or more cutoff values; and based on the comparison, determining a classification of whether a fetal chromosomal aneuploidy exists for the first chromosome.

56. The method of claim 55, wherein the sequencing is performed randomly on a portion of the nucleic acid molecules contained in the biological sample.

57. The method of claim 55, wherein the biological sample is maternal blood, plasma, serum, urine or saliva.

58. The method of claim 55, wherein the biological sample is transcervical lavage fluid.

59. The method of claim **55**, wherein the first chromosome is chromosome 21, chromosome 18, chromosome 13, chromosome X, or chromosome Y.

60. The method of claim **55**, wherein the parameter is a ratio of sequences that originate from the first chromosome.

61. The method of claim **60**, wherein the ratio is obtained from any one or more of a fractional count of the number of sequenced tags, a fractional number of sequenced nucleotides, and a fractional length of accumulated sequences.

62. The method of claim **60**, wherein the sequences that originate from the first chromosome are selected to be less than a specified number of base pairs.

63. The method of claim **62**, wherein the specified number of base pairs is 300 bp, 200 bp, or 100 bp.

64. The method of claim **55**, wherein the nucleic acid molecules of the biological sample have been enriched for sequences originating from at least one particular chromosome.

65. The method of claim **55**, wherein the nucleic acid molecules of the biological sample have been enriched for sequences less than 300 bp.

66. The method of claim **55**, wherein the nucleic acid molecules of the biological sample have been enriched for sequences less than 200 bp.

67. The method of claim **55**, wherein the nucleic acid molecules of the biological sample have been amplified using a polymerase chain reaction.

68. The method of claim **55**, wherein the sequenced portion represents at least a pre-determined fraction of the human genome.

69. The method of claim **55**, wherein the fraction represents at least 0.1% of the human genome.

70. The method of claim **55**, wherein the fraction represents at least 0.5% of the human genome.

71. The method of claim **55**, wherein at least one of the cutoff values is related to the fractional concentration of fetal DNA in the biological sample.

72. The method of claim **71**, wherein the fractional concentration of fetal DNA in the biological sample is determined by any one or more of a proportion of Y chromosome sequences, a fetal epigenetic marker, or using single nucleotide polymorphism analysis.

73. The method of claim **55**, wherein a cutoff value is a reference value established in a normal biological sample.

74. The method of claim **55**, further comprising: identifying an amount of fetal DNA in the biological sample; and calculating a number N of sequences to be analyzed based on a desired accuracy.

75. A computer program product comprising a computer readable medium encoded with a plurality of instructions for controlling a computing system to perform an operation for performing prenatal diagnosis of a fetal chromosomal aneuploidy in a biological sample obtained from a pregnant female subject, wherein the biological sample includes nucleic acid molecules, the operation comprising the steps of: receiving data from a random sequencing of a portion of the nucleic acid molecules contained in the biological sample obtained from a pregnant female subject, wherein the biological sample includes nucleic acid molecules, wherein the portion represents a fraction of the human genome; based on the data from the random sequencing: determining a first amount of a first chromosome from sequences identified as originating from the first chromosome; determining a second amount of one or more second chromosomes from sequences identified

as originating from one of the second chromosomes; determining a parameter from the first amount and the second amount; comparing the parameter to one or more cutoff values; and based on the comparison, determining a classification of whether a fetal chromosomal aneuploidy exists for the first chromosome.

76. A method for performing prenatal diagnosis of a fetal chromosomal aneuploidy in a biological sample obtained from a pregnant female subject, wherein the biological sample includes nucleic acid molecules, the method comprising: receiving the biological sample; calculating a number N of sequences to be analyzed based on a desired accuracy; randomly sequencing at least N of the nucleic acid molecules contained in the biological sample, wherein the portion represents a fraction of the human genome; based on the random sequencing: determining a first amount of a first chromosome from sequences identified as originating from the first chromosome; determining a second amount of one or more second chromosomes from sequences identified as originating from one of the second chromosomes; determining a parameter from the first amount and the second amount; comparing the parameter to one or more cutoff values; and based on the comparison, determining a classification of whether a fetal chromosomal aneuploidy exists for the first chromosome.

77. The method of claim **76**, further comprising: identifying a percentage of fetal DNA in the biological sample, wherein calculating a number N of sequences to be analyzed based on a desired accuracy is based on the percentage.

78. A method for performing prenatal diagnosis of a fetal chromosomal aneuploidy in a sample from a human suspected of being pregnant, that is pregnant, or that has been pregnant, wherein the sample includes nucleic acids, the method comprising: obtaining the sample; sequencing nucleic acids in the sample to produce partial or complete genome sequence; based on the sequencing: quantifying DNA from a chromosome suspected of being aneuploid; quantifying DNA from one or more second chromosomes; combining DNA quantity information from each chromosome; comparing the information to a pre-determined value; and based on the comparison, determining the presence or absence of fetal aneuploidy.

79. The method of claim **78**, wherein said sequencing is ultra-deep sequencing.

80. The method of claim **78**, wherein said maternal sample is maternal blood, urine, or saliva.

81. The method of claim **78**, wherein said maternal sample is a genitourinary tract fluid.

82. The method of claim **78**, wherein the first chromosome is chromosome 21, chromosome 18, chromosome 13, chromosome X, or chromosome Y.

83. The method of claim **78**, wherein the combined information comprises a ratio of sequences that originate from the chromosome suspected of being aneuploid.

84. The method of claim **83**, wherein the ratio is obtained by determining abundance of selected alleles.

85. The method of claim **83**, wherein the sequences from the first chromosome are up to a specified number of base pairs.

86. The method of claim **85**, wherein the specified number of base pairs is 300 bp, 200 bp, or 100 bp.

87. The method of claim **78**, wherein the nucleic acid molecules of the maternal sample have been enriched for sequences originating from at least one chromosome.

88. The method of claim **78**, wherein the nucleic acid molecules of the maternal sample have been enriched for sequences up to 300 bp.

89. The method of claim **78**, wherein the nucleic acid molecules of the maternal sample have been enriched for sequences up to 200 bp.

90. The method of claim **78**, wherein the nucleic acid molecules of the maternal sample have been enriched by polymerase chain reaction.

91. The method of claim **78**, wherein the sequenced portion represents at least a pre-determined number of chromosomal loci.

92. The method of claim **78**, wherein the total number of chromosomal loci is more than 100,000, wherein each loci comprises up to 30 bp.

93. The method of claim **78**, wherein the total number of chromosomal loci is more than 500,000, wherein each loci comprises up to 30 bp.

94. The method of claim **78**, wherein fetal aneuploidy determination involves a step of determining fetal/maternal DNA copy ratio.

95. The method of claim **94**, wherein the fetal/maternal DNA copy ratio is determined using small nucleotide polymorphisms.

96. The method of claim **78**, wherein said comparison used for fetal aneuploidy determination corresponds to a ratio of alleles in a normal sample.

97. The method of claim **78**, further comprising determining an amount of allele abundance in a sample; and calculating a number of sequence reads required to be analyzed to detect ploidy changes with high levels of confidence.

98. A computer program comprising a computer executable logic recorded on a computer readable medium configured to support and execute functions for determining a prenatal diagnosis of a fetal chromosomal aneuploidy in a sample from a human suspected of being pregnant, that is pregnant, or that has been pregnant, wherein the sample includes nucleic acids, including acts of receiving data from the one or more genomic DNA regions analyzed by sequencing nucleic acids in the sample to produce partial or complete genome sequence; based on the sequencing: quantifying DNA from a chromosome suspected of being aneuploid; quantifying DNA from one or more second chromosomes; combining DNA quantity information from each chromosome; comparing the information to a pre-determined value; and based on the comparison, determining the presence or absence of fetal aneuploidy.

99. A method for performing prenatal diagnosis of a fetal chromosomal aneuploidy in a sample from a human suspected of being pregnant, that is pregnant, or that has been pregnant, wherein the sample includes nucleic acids, the method comprising

obtaining the sample; calculating a number of sequence reads required to be analyzed to detect ploidy changes with high levels of confidence;

sequencing nucleic acids in the sample to produce partial or complete genome sequence;

obtaining the number of sequence reads required to be analyzed to detect ploidy changes with high levels of confidence; based on the sequencing:

quantifying DNA from a chromosome suspected of being aneuploid;

quantifying DNA from one or more second chromosomes;

combining information from each chromosome;

comparing the information to a pre-determined value;

and based on the comparison, determining the presence or absence of fetal aneuploidy.

100. The method of claim **99**, further comprising determining the fetal/maternal DNA copy ratio.

* * * * *

专利名称(译)	使用样品分裂和DNA标签进行稀有细胞分析		
公开(公告)号	US20100136529A1	公开(公告)日	2010-06-03
申请号	US12/230628	申请日	2008-09-02
[标]申请(专利权)人(译)	通用医疗公司 GPB科学有限责任公司 维里纳塔健康公司		
申请(专利权)人(译)	总医院CORPORATION GPB科技有限责任公司 VERINATA HEALTH，INC.		
当前申请(专利权)人(译)	VERINATA HEALTH，INC.		
[标]发明人	SHOEMAKER DANIEL FUCHS MARTIN KRUEGER NEIL X TONER MEHMET GRAY DARREN KAPUR RAVI WANG ZIHUA		
发明人	SHOEMAKER, DANIEL FUCHS, MARTIN KRUEGER, NEIL X. TONER, MEHMET GRAY, DARREN KAPUR, RAVI WANG, ZIHUA		
IPC分类号	C12Q1/68 G01N33/53 G06N5/02		
CPC分类号	B01L3/502761 C12Q1/6809 C12Q1/6883 C12Q1/6869 C12Q1/6881 Y10T436/143333 C12Q2600/158 C12Q2600/16 G01N2015/1006 G01N2015/1087 C12Q2600/156		
优先权	60/804819 2006-06-14 US		
其他公开文献	US8168389		
外部链接	Espacenet USPTO		

摘要(译)

本发明提供了当与样品中的一组母体细胞混合时检测胎儿细胞的存在并测试胎儿异常的系统，装置和方法，例如，非整倍体。本发明涉及用不同标记标记所述混合样品中每个细胞中基因组DNA的区域，其中每个标记对每个细胞是特异性的，并定量混合样品中每个细胞的基因组DNA的标记区域。更具体地，本发明涉及从混合样品中的每个细胞中定量标记的DNA多态性。

Average
Flow
Direction

Lateral
Flow
Direction

$d = 8\ \mu m$

$12\ \mu m$