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HAHN et al.(10) **Pub. No.: US 2012/0302741 A1**(43) **Pub. Date: Nov. 29, 2012**(54) **NON-INVASIVE DETECTION OF FETAL
GENETIC TRAITS**(75) Inventors: **Sinuhe HAHN**, Liestal (CH);
Wolfgang Holzgreve, Basel (CH);
Bernhard Zimmermann, Los
Angeles, CA (US); **Ying Li**, Basel
(CH)(73) Assignee: **SEQUENOM, INC.**, San Diego,
CA (US)(21) Appl. No.: **13/557,025**(22) Filed: **Jul. 24, 2012****Related U.S. Application Data**(63) Continuation of application No. 10/964,726, filed on
Oct. 15, 2004, now abandoned.(30) **Foreign Application Priority Data**

Oct. 16, 2003 (EP) 03405742.2

Publication Classification(51) **Int. Cl.**
C07H 21/04 (2006.01)(52) **U.S. Cl.** **536/24.33; 536/23.1**(57) **ABSTRACT**

Blood plasma of pregnant women contains fetal and (generally >90%) maternal circulatory extracellular DNA. Most of said fetal DNA contains ≤ 500 base pairs, said maternal DNA having a greater size. Separation of circulatory extracellular DNA of <500 base pairs results in separation of fetal from maternal DNA. A fraction of a blood plasma or serum sample of a pregnant woman containing, due to size separation (e.g. by chromatography, density gradient centrifugation or nanotechnological methods), extracellular DNA substantially comprising ≤ 500 base pairs is useful for non-invasive detection of fetal genetic traits (including the fetal RhD gene in pregnancies at risk for HDN; fetal Y chromosome-specific sequences in pregnancies at risk for X chromosome-linked disorders; chromosomal aberrations; hereditary Mendelian genetic disorders and corresponding genetic markers; and traits decisive for paternity determination) by e.g. PCR, ligand chain reaction or probe hybridization techniques, or nucleic acid arrays.

NON-INVASIVE DETECTION OF FETAL GENETIC TRAITS

RELATED APPLICATIONS

[0001] This patent application is a continuation of U.S. Patent Application No. 10/964,726 filed on Oct. 15, 2004, entitled NON-INVASIVE DETECTION OF FETAL GENETIC TRAITS, naming Sinuhe Hahn, Wolfgang Holzgreve, Bernhard Zimmermann, and Ying Li as inventors, and designated by Attorney Docket No. SEQ-5002-UT, which claims the benefit under 35 U.S.C. 119(a) of European Patent Application No. 03405742.2 filed on Oct. 16, 2003, entitled NON-INVASIVE DETECTION OF FETAL GENETIC TRAITS, naming Sinuhe Hahn, Wolfgang Holzgreve, Bernhard Zimmermann, and Ying Li as inventors and designated by Attorney Docket No. SEQ-5002-EP. The entirety of each of these patent applications is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The presence of circulatory extracellular DNA in the peripheral blood is a well established phenomenon. In this context, it has been shown that in the case of a pregnant woman extracellular fetal DNA is present in the maternal circulation and can be detected in maternal plasma or serum. Studies have shown that this circulatory fetal genetic material can be used for the very reliable determination, e.g. by PCR (polymerase chain reaction) technology, of fetal genetic loci which are completely absent from the maternal genome. Examples of such fetal genetic loci are the fetal RhD gene in pregnancies at risk for HDN (hemolytic disease of the fetus and newborn) or fetal Y chromosome-specific sequences in pregnancies at risk for an X chromosome-linked disorder e.g. hemophilia or fragile X syndrome.

[0003] The determination of other, more complex fetal genetic loci (e.g. chromosomal aberrations such as aneuploidies or chromosomal aberrations associated with Down's syndrome, or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith, such as single gene disorders, e.g. cystic fibrosis or the hemoglobinopathies) is, however, more problematic. The reason for this difficulty is that the major proportion (generally >90%) of the extracellular DNA in the maternal circulation is derived from the mother. This vast bulk of maternal circulatory extracellular DNA renders it difficult, if not impossible, to determine fetal genetic alterations such as those involved in chromosomal aberrations (e.g. aneuploidies) or hereditary Mendelian genetic disorders (e.g. cystic fibrosis or the hemoglobinopathies) from the small amount of circulatory extracellular fetal DNA.

SUMMARY OF THE INVENTION

[0004] An examination of circulatory extracellular fetal DNA and circulatory extracellular maternal DNA in maternal plasma has now shown that, surprisingly, the majority of the circulatory extracellular fetal DNA has a relatively small size of approximately 500 base pairs or less, whereas the majority of circulatory extracellular maternal DNA in maternal plasma has a size greater than approximately 500 base pairs. Indeed, in certain instances the circulatory DNA material which is smaller than approximately 500 base pairs appears to be almost entirely fetal. Circulatory extracellular fetal DNA in the maternal circulation has thus been found to be smaller in

size (approximately 500 base pairs or less) than circulatory extracellular maternal DNA (greater than approximately 500 base pairs).

[0005] This surprising finding forms the basis of the present invention according to which separation of circulatory extracellular DNA fragments which are smaller than approximately 500 base pairs provides a possibility to enrich for fetal DNA sequences from the vast bulk of circulatory extracellular maternal DNA.

[0006] This selective enrichment, which is based on size discrimination of circulatory DNA fragments of approximately 500 base pairs or less, leads to a fraction which is largely constituted by fetal extracellular DNA. This permits the analysis of fetal genetic traits including those involved in chromosomal aberrations (e.g. aneuploidies or chromosomal aberrations associated with Down's syndrome) or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith (e.g. single gene disorders such as cystic fibrosis or the hemoglobinopathies), the determination of which had, as mentioned above, so far proved difficult, if not impossible. Size separation of extracellular fetal DNA in the maternal circulation thus facilitates the non-invasive detection of fetal genetic traits, including paternally inherited polymorphisms which permit paternity testing.

[0007] Clinical Chemistry, 1999, Vol. 45(9), pages 1570-1572 and The Australian & New Zealand

[0008] Journal of Obstetrics & Gynaecology, February 2003 (O.sub.2-2003), Vol. 43(1), pages 10-15 describe a sample of blood plasma of a pregnant woman in which extracellular fetal DNA of less than 500 base pairs is enriched by PCR, is separated by gel electrophoresis and fetal male DNA (fetal Y-chromosome-specific sequence) is detected.

[0009] The present invention provides: a fraction of a sample of the blood plasma or serum (which preferably is substantially cell-free) of a pregnant woman in which, as the result of said sample having been submitted to a size separation, the extracellular DNA present therein substantially consists of DNA comprising 500 base pairs or less; the use of such sample-fraction for the non-invasive detection of fetal genetic traits; and a process for performing non-invasive detection of fetal genetic traits which comprises subjecting a sample of the blood plasma or serum of a pregnant woman to a size separation so as to obtain a fraction of said sample in which the extracellular DNA present therein substantially consists of DNA comprising 500 base pairs or less, and determining in said sample-fraction the fetal genetic trait(s) to be detected.

[0010] Said serum or plasma sample is preferably substantially cell-free, and this can be achieved by known methods such as, for example, centrifugation or sterile filtration.

[0011] The size separation of the extracellular DNA in said serum or plasma sample can be brought about by a variety of methods, including but not limited to: chromatography or electrophoresis such as chromatography on agarose or polyacrylamide gels, ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC, see Hecker K H, Green S M, Kobayashi K, J. Biochem. Biophys. Methods 2000 Nov. 20; 46(1-2): 83-93), capillary electrophoresis in a self-coating, low-viscosity polymer matrix (see Du M, Flanagan J H Jr, Lin B, Ma Y, Electrophoresis 2003 September; 24 (18): 3147-53), selective extraction in microfabricated electrophoresis devices (see Lin R, Burke D T, Burn M A, J. Chromatogr. A. 2003 Aug. 29; 1010(2): 255-68), microchip electrophoresis on reduced viscosity polymer matrices (see Xu F,

Jabasini M, Liu S, Baba Y, *Analyst*. 2003 June; 128(6): 589-92), adsorptive membrane chromatography (see Teeters MA, Conrardy S E, Thomas B L, Root T W, Lightfoot E N, J. *Chromatogr. A*. 2003 Mar. 7; 989(1): 165-73) and the like; density gradient centrifugation (see Raptis L, Menard H A, J. *Clin. Invest.* 1980 December; 66(6): 1391-9); and methods utilising nanotechnological means such as microfabricated entropic trap arrays (see Han J, Craighead H G, *Analytical Chemistry*, Vol. 74, No. 2, Jan. 15, 2002) and the like.

[0012] The sample-fraction thus obtained not only permits the subsequent determination of fetal genetic traits which had already been easily detectable in a conventional manner such as the fetal RhD gene in pregnancies at risk for HDN (hemolytic disease of the fetus and the newborn), or fetal Y chromosome-specific sequences in pregnancies at risk for an X chromosome-linked disorder such as hemophilia, fragile X syndrome or the like, but also the determination of other, more complex fetal genetic loci, including but not limited to: chromosomal aberrations (e.g. aneuploidies or Down's syndrome) or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith (e.g. single gene disorders such as cystic fibrosis or the hemoglobinopathies); and fetal genetic traits which may be decisive when paternity is to be determined.

[0013] Such determination of fetal genetic traits can be effected by methods such as, for example, PCR (polymerase chain reaction) technology, ligase chain reaction, probe hybridization techniques, nucleic acid arrays (so-called "DNA chips") and the like.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0014] The following Examples further illustrate the invention but are not to be construed as limiting its scope in any way.

EXAMPLE 1

Detection of Male Fetal DNA in Maternal Plasma by Real-Time Quantitative Polymerase Chain Reaction (PCR) After Size Fractionation of DNA by Agarose Gel Electrophoresis

Materials and Methods

[0015] Subjects and Sample Processing

[0016] Seven women pregnant in the third trimester with a male fetus were recruited for this study. 16-18 ml blood samples were collected into EDTA tubes. 6-9 ml of plasma were obtained after centrifugation at 1600 g for 10 minutes and a second centrifugation of the supernatant at 16000 g for 10 minutes.

[0017] DNA Isolation

[0018] DNA from 5-7 ml plasma was extracted using the QIAgen Maxi kit, according to the manufacturers' protocol. DNA was eluted in a volume of 1.5 ml.

[0019] DNA Precipitation

[0020] 1. To the plasma DNA were added: 1/10 volume NaAc (3M, pH 5.2), 2 volumes absolute ethanol, $MgCl_2$ to a final concentration of 0.01 M and Glycogen to a final concentration of 50 µg/ml. The solution was thoroughly mixed by vortexing.

[0021] 2. The solution was stored overnight at -70° C.

[0022] 3. The DNA was recovered by centrifugation at 20000 g for 30 minutes at 4° C.

[0023] 4. The supernatant was carefully removed and the pellet washed with 500 µl 70% ethanol.

[0024] 5. The pellet was air dried and dissolved in 35 µl distilled water.

[0025] DNA Separation

[0026] 1. A 1% agarose Gel (Invitrogen, Cat No: 15510-027) was prepared for DNA electrophoresis.

[0027] 2. 28 µl DNA solution were loaded on the gel.

[0028] 3. The gel was electrophoresed at 80 Volt for 1 hour.

[0029] 4. The Gel was cut into pieces corresponding to specific DNA sizes according to the DNA size markers (New England Biolabs, 100 bp ladder and Lambda Hind III digest). The DNA sizes contained by the specific gel fragments were: 90-300 bases, 300-500 bases, 500-1000 bases, 1.0-1.5 kilobases ("kb"), 1.5-23 kb and >23 kb.

[0030] 5. The DNA was purified from the agarose gel pieces using the QIAEX II Gel Extraction kit (Qiagen, Cat No. 20021) and eluted in 35 µl Tris-HCl (pH 8.0, 10 mM).

[0031] Real-Time PCR

[0032] Sequences from the Y chromosome (SRY) and from chromosome 12 (GAPDH gene) were amplified with the Applied Biosystems (ABI) 7000 Sequence Detection System by real-time quantitative PCR to quantify amounts of fetal and total DNA in the size-separated fractions. The TaqMan system for SRY consisted of the amplification primers SRY_Fwd: TCC TCA AAA GAA ACC GTG CAT (SEQ ID NO: 1) and SRY Rev: AGA TTA ATG GTT GCT AAG GAC TGG AT (SEQ ID NO: 2) and a FAM labeled TaqMan MGB (Minor Groove Binder) probe SRY_MGB: TCC CCA CAA CCT CTT (SEQ ID NO: 3). The TaqMan System for the GAPDH gene consisted of the following primers and probe: GAPDH_Fwd: CCC CAC ACA CAT GCA CTT ACC (SEQ ID NO: 4), GAPDH_Rev: CCT AGT CCC AGG GCT TTG ATT (SEQ ID NO: 5) and GAPDH_MGB: TAG GAA GGA CAG GCA AC (SEQ ID NO: 6).

[0033] TaqMan amplification reactions were set up in a total reaction volume of 25 µl, containing 6 µl of the sample DNA solution, 300 nM of each primer (HPLC purified, Mycosynth, Switzerland) and 200 nM of each probe (ABI) at 1x concentration of the Universal PCR reaction mix (ABI). Each sample was analyzed in duplicate for each of the two amplification systems. A standard curve containing known amounts of genomic DNA was run in parallel with each analysis.

[0034] Thermal cycling was performed according to the following protocol: an initial incubation at 50° C. for 2 minutes to permit Amp Erase activity, 10 minutes at 95° C. for activation of AmpliTaq Gold, and 40 cycles of 1 minute at 60° C. and 15 seconds at 95° C. Amplification data collected by the 7000 Sequence Detection System was quantified using the slope of the standard curve as calculated by the sequence detection software and the results of a standard DNA solution used in the dilution curve with similar DNA copy numbers as the sample reactions as a reference sample for copy number calculations.

Results

[0035] Table 1 shows that in the five pregnancies examined, DNA fragments originating from the fetus were almost completely of sizes smaller than 500 base pairs with around 70% being of fetal origin for sizes smaller than 300 bases.

[0036] These results demonstrate that free DNA of fetal origin circulating in the maternal circulation can be specifically enriched by size separation of the total free DNA in the maternal blood.

[0037] Depending on the downstream application the DNA size chosen for the enrichment of fetal DNA will be smaller than 300 or smaller than 500 bases.

TABLE 1

Size of DNA	% of fetal DNA in each fragment	% of maternal DNA in each fragment
<0.3 kb	73.2 (22.22-87.06)	26.8 (12.94-77.78)
0.3-0.5 kb	18.95 (6.43-31.42)	81.05 (68.58-93.57)
0.5-1 kb	2.81 (0.00-7.75)	97.19 (92.25-100)
1.0-1.5 kb	0.00 (0.00-12.50)	100 (87.5-100)
1.5-23 kb	0.00 (0.00-8.40)	100 (100-100)

[0038] The abbreviation “kb” appearing in the first column of this table stands for 1000 base pairs, and the figures given in its second and the third column are the median values of the percentages and, in brackets, the ranges.

EXAMPLE 2

Detection of Fetal DNA After Agarose Gel Electrophoresis by Polymerase Chain Reaction (PCR) of Microsatellite Markers, also Called “Short Tandem Repeats” (STRs)

Materials and Methods

[0039] Subjects and Samples

[0040] 18 ml blood samples from pregnant women and 9 ml blood from their partners were collected into EDTA tubes and plasma separated by centrifugation as described in Example 1. The maternal buffy coat (i.e. the white colored top layer of the cell pellet obtained after the first centrifugation of 1600 g for 10 min.) was washed twice with PBS.

[0041] DNA Isolation

[0042] DNA from the plasma was extracted using a modification of the High Pure DNA template kit from Roche, the whole sample was passed through the filter usually used for 200 µl using a vacuum. The DNA was eluted in a volume of 50 µl elution buffer. Paternal DNA was extracted from 400 µl paternal whole blood, using the High Pure DNA template kit, and eluted into 100 µl. Maternal DNA was isolated from the buffy coat, using the High Pure DNA template kit, and eluted into 100 µl.

[0043] DNA Separation

[0044] The DNA was size-separated by electrophoresis on an agarose gel and purified as described in Example 1.

[0045] PCR Specific for Short Tandem Repeats

[0046] From the fraction of sizes smaller than 500 bases, sequences from tetranucleotide repeat markers on Chromosome 21 were amplified in a multiplex PCR reaction as

described in Li et al. Clinical Chemistry 49, No. 4, 2003. Because of the low concentration of plasma DNA, the fetal DNA in maternal plasma was examined by using a semi-nested PCR protocol.

[0047] The maternal and paternal pairs were genotyped using total genomic DNA to monitor microsatellite markers on chromosome 21.

[0048] The STR markers used were:

[0049] D211 S11;

[0050] D21S1270;

[0051] D21S1432; and

[0052] D21S1435

[0053] The resulting DNA fragments were then size separated by capillary electrophoresis on a sequencer, and the peak areas representing each allele for a specific marker were measured by the software.

Results

[0054]

TABLE 2

Detection of fetal alleles specific for the microsatellite marker (Short Tandem Repeat) D21S11 on chromosome 21		
	Maternal alleles detected (D21S11)	Fetal alleles detected (D21S11)
Maternal genomic DNA	232 bp	N/A
Total extracellular DNA (unseparated)	232 bp	No fetal alleles detectable
Size-separated extracellular DNA (<300 bp)	234 bp	
Size-separated extracellular DNA (300-500 bp)	232 bp	228 bp
	234 bp	232 bp

[0055] Only in the size-separated fractions (<300 by and 300-500 bp) could the fetal alleles for D21S11 be detected, namely the paternally inherited 228 by allele and the maternally inherited 232 by allele, i.e., one allele from each parent.

[0056] Discussion

[0057] Analysis of the STR fragments can allow for the detection of paternal alleles that are distinct in length from the maternal repeat sequences, and by calculating the ratios between the peak areas it can be possible to identify patterns that are not consistent with a normal fetal karyotype. The identification of paternal allele sizes of STRs in the maternal circulation can allow the detection of certain chromosomal aberrations non-invasively. Also paternity testing can be accomplished prenatal in a non-invasive manner.

SEQUENCE LISTING

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<210> SEQ ID NO 1

<211> LENGTH: 21

-continued

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:Primer
SRY_Fwd

<400> SEQUENCE: 1
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<210> SEQ ID NO 2
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:Primer
SRY_Rev

<400> SEQUENCE: 2
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<210> SEQ ID NO 3
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:FAM labeled
TaqMan MGB probe SRY_MGB

<400> SEQUENCE: 3
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<210> SEQ ID NO 4
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<400> SEQUENCE: 4
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<210> SEQ ID NO 5
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GAPDH_Rev

<400> SEQUENCE: 5
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:Probe
GAPDH_MGB

<400> SEQUENCE: 6
taggaaggac aggcaac 17

What is claimed is:

1. Isolated deoxyribonucleic acid (DNA), consisting of: DNA fragments of substantially 500 base pairs or less comprising a fraction of DNA from blood plasma of a pregnant female largely constituted of circulating extracellular fetal DNA,
which DNA fragments are not in association with a gel, and
which circulating extracellular fetal DNA in the isolated DNA is enriched relative to circulating extracellular DNA in the blood plasma of the pregnant female.
2. The isolated DNA of claim 1, wherein the DNA fragments are substantially 300 base pairs or less.
3. The isolated DNA of claim 2, wherein the DNA fragments are at least about 70% circulating extracellular fetal DNA.
4. The isolated DNA of claim 1, wherein the fetal DNA comprises an allele different than an allele in the maternal DNA.
5. The isolated DNA of claim 1, wherein the fetal DNA comprises a chromosome aneuploidy.
6. The isolated DNA of claim 1, wherein the fetal nucleic acid comprises a paternally inherited polymorphism not present in a maternally inherited sequence.
7. The composition of claim 6, wherein the paternally inherited polymorphism is of a length different than in the maternally inherited sequence.
8. The isolated DNA of claim 1, wherein the DNA fragments comprise DNA that can amplify DNA fragments in the isolated DNA.
9. The isolated DNA of claim 8, wherein the DNA fragments comprise a DNA primer that can amplify DNA fragments in the isolated DNA.
10. The isolated DNA of claim 1, wherein the DNA fragments are in solution.
11. Isolated deoxyribonucleic acid (DNA), consisting of: DNA fragments of substantially 500 base pairs or less comprising a fraction of DNA from blood plasma of a pregnant female largely constituted of circulating extracellular fetal DNA,

- which DNA fragments are not in association with a gel,
which circulating extracellular fetal DNA in the isolated DNA is enriched relative to circulating extracellular DNA in the blood plasma of the pregnant female, and
which isolated DNA is prepared by a process comprising:
- (a) extracting circulating extracellular DNA from the plasma from the pregnant female, thereby providing extracted DNA; and
 - (b) separating and isolating the extracted DNA by size, thereby providing separated and isolated DNA;
- whereby the separated and isolated DNA is enriched for the circulating extracellular fetal DNA.
12. The isolated DNA of claim 11, wherein the DNA fragments are substantially 300 base pairs or less.
 13. The isolated DNA of claim 12, wherein the DNA fragments are at least about 70% circulating extracellular fetal DNA.
 14. The isolated DNA of claim 11, wherein the fetal DNA comprises an allele different than an allele in the maternal DNA.
 15. The isolated DNA of claim 11, wherein the fetal DNA comprises a chromosome aneuploidy.
 16. The isolated DNA of claim 11, wherein the DNA fragments comprise DNA that can amplify DNA fragments in the isolated DNA.
 17. The isolated DNA of claim 16, wherein the DNA fragments comprise a DNA primer that can amplify DNA fragments in the isolated DNA.
 18. The isolated DNA of claim 11, wherein the DNA fragments are in solution.
 19. The isolated DNA of claim 11, wherein separating the DNA in step (b) comprises electrophoresis.
 20. The isolated DNA of claim 11, wherein separating the DNA in step (b) comprises chromatography.

* * * * *

专利名称(译)	胎儿遗传性状的非侵入性检测		
公开(公告)号	US20120302741A1	公开(公告)日	2012-11-29
申请号	US13/557025	申请日	2012-07-24
[标]申请(专利权)人(译)	塞昆纳姆股份有限公司		
申请(专利权)人(译)	SEQUENOM INC.		
当前申请(专利权)人(译)	SEQUENOM , INC		
[标]发明人	HAHN SINUHE HOLZGREVE WOLFGANG ZIMMERMANN BERNHARD LI YING		
发明人	HAHN, SINUHE HOLZGREVE, WOLFGANG ZIMMERMANN, BERNHARD LI, YING		
IPC分类号	C07H21/04 G01N33/53 B01J20/281 C12N15/09 C12Q1/68 G01N30/88 G01N37/00		
CPC分类号	C12Q1/6806 C12Q2600/156 C12Q1/6883 C12Q2565/125		
优先权	2003405742 2003-10-16 EP		
外部链接	Espacenet USPTO		

摘要(译)

孕妇的血浆含有胎儿和 (通常> 90%) 母体循环细胞外DNA。大多数所述胎儿DNA含有≤500个碱基对, 所述母体DNA具有更大的大小。分离<500碱基对的循环细胞外DNA导致胎儿与母体DNA分离。由于大小分离 (例如通过色谱, 密度梯度离心或纳米技术方法), 包含基本上包含≤500碱基对的细胞外DNA的孕妇血浆或血清样品的一部分可用于胎儿遗传的非侵入性检测特征 (包括有妊娠HDN风险的胎儿RhD基因; X染色体相关疾病风险的妊娠胎儿Y染色体特异性序列;染色体畸变;遗传性孟德尔遗传疾病和相应的遗传标记;以及确定亲子鉴定特征) 通过例如PCR, 配体链反应或探针杂交技术, 或核酸阵列。

Size of DNA	% of fetal DNA in each fragment	% of maternal DNA in each fragment
<0.3 kb	73.2 (22.22-87.06)	26.8 (12.94-77.78)
0.3-0.5 kb	18.95 (6.43-31.42)	81.05 (68.58-93.57)
0.5-1 kb	2.81 (0.00-7.75)	97.19 (92.25-100)
1.0-1.5 kb	0.00 (0.00-12.50)	100 (87.5-100)
1.5-23 kb	0.00 (0.00-8.40)	100 (100-100)