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Analysis of fetal DNA in maternal plasma with markers designed for forensic DNA mixture resolution

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DISCLOSURE

The authors declare no conflict of interest.

ABSTRACT

Purpose: With the description of circulating fetal DNA in maternal blood, non-invasive prenatal diagnostics became theoretically possible. As the presence of background maternal DNA interferes with the detection of fetal DNA, analytical methods require genetic markers capable of distinguishing by quantitative or targeted approaches the minor population of DNA molecules of the fetus. Here we evaluate the feasibility of analyzing fetal DNA with novel DIP-STR genetic markers, designed for the investigation of forensic mixed biological evidence.

Methods: The DIP-STR molecular approach is based on sequence-specific analysis of paternally inherited fetal alleles. These sequences are biallelic deletion/insertion polymorphisms (DIP) located very close to STR markers, for combined analysis. In this study, 48 women were tested with 28 DIP-STRs during the first, second and third trimester of pregnancy.

Results: Positive results were obtained across markers, including longer ones (386 base-pairs) and with blood samples collected during early pregnancy, such as ten weeks of gestational age.

Conclusions: These data show that DIP-STR markers can be used to amplify specific genomic regions of circulating fetal DNA to obtain targeted genetic information. This method may contribute to developments in non-invasive prenatal paternity testing and diagnosis of certain genetic diseases.

Key Words noninvasive prenatal testing; paternity testing; cell-free DNA; DIP-STR; DNA mixture

INTRODUCTION

In obstetric care, prenatal screening and diagnosis allows women to make informed choices about the continuation of the pregnancy affected by genetic conditions such as: sex-linked disorders,[1] fetal RhD status,[2,3] β-thalassemia,[4-6] congenital adrenal hyperplasia,[7] chromosomal aneuploidy,[8,9] pre-eclampsia[10,11] and others.[12] In forensic science, prenatal paternity testing aims at helping those pregnant women who have been victims of sexual assault and are unclear about the paternity of their unborn child.

Unfortunately, many definitive prenatal diagnoses of diseases including reliable prenatal paternity tests require the analysis of fetal genetic material, obtained through procedures performed at advanced gestational age (about 15 weeks) and that are associated to a certain degree of harm to the mother and the fetus. For instance, amniocentesis carries a miscarriage risk of 1 in 300 to 1 in 500[13] and a small risk of other complications,[14,15] direct chorionic villus sampling carries a similar miscarriage risk in addition to a 1 in 3000 risk of fetal limb reduction defects, especially when performed before the 10th week of gestation.[16,17]

New possibilities of developing prenatal diagnostic methods that are noninvasive, emerged with the discovery of the presence of fetal DNA in maternal blood by Lo and collaborators.[18] The continuous remodeling of the placenta, with trophoblast cells undergoing apoptotic events, is responsible of the releasing of cell-free fetal DNA (cffDNA) in the blood stream of the mother. Because of its apoptotic origin, cffDNA is mostly short (about 200 base-pairs), it appears at around six weeks of pregnancy and it constitutes approximately 10% of the total cell free plasma DNA,[19-21] thus generating an *in vivo* DNA mixture characterized by a large excess of maternal DNA (DNA microchimerism).[22,23] Conventional DNA sequencing, SNPs and STRs markers based on PCR and capillary electrophoresis are not sensitive enough for characterizing a DNA contributing less than 10% to a mixed sample and sex-specific Y-STR markers cannot be used in

case of baby girls and to distinguish paternally related alleged fathers. Unless progress is made in methods for the enrichment of the fetal DNA fraction;[24] one possibility for the analysis of the DNA microchimerism in pregnancy is the use of high-precision approaches. Digital PCR, [25] large SNP array[26] and massive parallel sequencing (MPS)[27] for example, enable to detect the small amount of sequences from a particular locus, even when applied to a sample in which fetal DNA only represents a small fraction.

Few studies evaluated the feasibility of non-invasive prenatal paternity testing by using high-throughput SNP genotyping.[28,29] In Ryan et al. 2013, an array of about 300,000 SNPs optimized to detect cytogenetic abnormalities most relevant to human disease is used to establish the genotype of the plasma DNA. The genetic information of the mother (reference sample) is used to identify a series of SNP alleles transmitted by the father and with a 'Paternal Support algorithm'[28] it is possible to estimate the probability of paternity of the alleged father. In addition to genotyping, this technique can be used to provide the relative quantity of alleles at each SNP to detect the presence or absence of fetal aneuploidies. Limitations of this approach include sometimes a low signal-to-noise ratio because the fetal DNA is neither enriched nor specifically targeted. Large-scale clinical trials are also needed to more accurately establish the specificity and sensitivity of the test.

Recently, MPS has also been used to analyse cffDNA in maternal plasma.[30] As above, the use of the maternal DNA as reference sample enables the identification of the fetal DNA and the maternal versus paternal DNA inheritance. Applications focused so far on the detection of aneuploidies, because the quantitative detection of the chromosomal dosage over an extended region is more feasible than single mutation analysis. For paternity testing,[31-34] the method currently lacks informatics based analysis necessary to evaluate the accuracy with which

paternity is confirmed. Signal-to-noise ratio is also an issue with many informative paternally transmitted alleles filtered out after quality controls.

Both these procedures, SNP array and MPS are labor intensive and require dedicated equipment. Another limitation associated to the genome wide screening of the fetal DNA is that the data gathered provide information beyond the specific genetic question investigated and may generate unclear and or undesired diagnostics.

To circumvent these problems, we developed a PCR-based method that uses allele-specific primers to target DNA sequences that are unique to the fetal DNA (transmitted by the father).[35,36] These sequences are biallelic deletion/insertion polymorphisms (DIP) of several nucleotides, mostly between three and 15. The two possible alleles are referred to as, long allele (L) and short allele (S). Because biallelic markers have reduced information content, we proposed the selection of DIP markers that are physically very close (linked) to another STR marker, for combined analysis. The newly generated compound markers are termed DIP-STRs (**Figure 1**). The multiallelic haplotype composed of both DIP and STR alleles is analyzed by using PCR primers overlapping the deleted/inserted sequence (S-DIP, L-DIP primers) on one side and downstream the STR region on the other side (STR primer). The sequence difference between S- and L-specific primers of several nucleotides allows the selective (allele-specific) amplification of the fetal DNA contributor in the presence of large quantities (more than 1,000 fold excess) of maternal DNA background. In this way, it is possible to target those paternally transmitted alleles that are not shared with the maternal DNA.

In this study, we determined whether the DIP-STR markers can be used to analyze cffDNA in maternal plasma for noninvasive prenatal diagnosis. The results from testing this new method on 48 pregnant women with 95 samples collected at different gestational ages, show that DIP-STRs

can contribute to prenatal care; especially considering the potential for improvement in marker selection, multiplexing and quantity of maternal plasma to be used for testing.

MATERIALS AND METHODS

Sample collection

Inclusion criteria for enrolled couples were singleton pregnancies with known paternity. Maternal blood samples (10ml) were drawn longitudinally from 48 women at 8-13 weeks (first trimester), 14-26 weeks (second trimester) and 27-40 weeks (third trimester) of amenorrhea. Samples collected during the first, second and third trimester are from 27, 28 and 40 pregnant women, respectively. See **Table 1** for details on longitudinal sample collection. Venous blood samples were drawn into EDTA blood collection tubes. Plasma was separated from the blood cells via double centrifugation (1,600 g for 10 min, tube transfer, and centrifugation at 28,000 g for 10 min) within 2 hours from blood drawn. Four to five aliquots of 1 ml were stored at -20°C until further processing.[37]

DNA samples from both parents of the developing baby were collected by buccal swab. Such reference samples are necessary for searching DIP-STR markers showing informative genotypes. Written informed consent was obtained from all participants, and genetic samples were collected under an institutional review board-approved research protocol.

DNA extraction

Cell free circulating DNA was extracted in duplicate from 2 ml of plasma by using the QIAamp Circulating Nucleic Acid Kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's protocol. Absorbed DNA was eluted with 60 µl of provided elution buffer. The synthetic DNA RT-SPCY-T02 (Eurogentec, Angers, France) was added to the plasma to function as positive control for circulating DNA extraction. According to the manufacturer's protocol, 2 ul of a 10

fold diluted RT-SPCY-T02 was added to 2 ml of plasma. Reference buccal samples were extracted using the QIAamp DNA Mini Kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's protocol and eluted in 100 µl final volume. Both genomic and circulating DNA samples were stored at -20°C.

Fetal DNA quantity and percent was measured in the subset of plasma samples from pregnancies of male fetuses. The forensic kit Investigator Quantiplex HYresTM assay (Qiagen AG, Basel, Switzerland) and an AB 7500 Real-time PCR system (Life Technologies Europe, Zug, Switzerland) was used according to the manufacturer's protocol, data were analyzed using the HID Real-Time PCR Analysis Software v1.2 (Life Technologies Europe, Zug, Switzerland).

DIP-STR genetic markers

The DIP-STR markers genotyped for this study include 18 markers previously published[35,36] and ten newly developed (**Supplementary Table S1**).

PCR reactions for the ten new markers were performed as previously published DIP-STR genotyping protocols[35,36] using 10 ul of plasma DNA. Primer sequences are reported in **Supplementary Table S2**. S- and L-DIP-STR specific amplifications were done in singleplex according to published protocols.[35,36] For the ten newly developed markers primers were used at 100 nM and 34 cycles of PCR. Annealing temperatures varied: rs71725104-STR, rs72534187-STR, rs139592446-STR, rs36194161-STR, rs138331044-STR were amplified at 58°C, MID473-STR, MID2538-STR, MID1739-STR, MID2824-STR at 55°C and MID73-STR at 59°C. To identify informative markers for plasma DNA analysis, reference DNA samples from the mothers and the fathers were first genotyped for 28 DIP markers using four multiplex reactions as described in **Supplementary Table S3**. For all markers PCR thermal cycling conditions were: 5 min at 95°C, 1 min at 94°C, 1 min at annealing temperature specific to the markers set to be

genotyped, 1 min at 72°C for a number of PCR cycles that also varied across multiplex and a final extension of 30 min at 72°C.

PCR fragments were separated by capillary electrophoresis after adding 1 µl PCR amplicon to 8.5 µl deionized formamide HI-DI (Life Technologies Europe, Zug, Switzerland) and to 0.5 µl 600 LIZ size standard (Life Technologies Europe, Zug, Switzerland). Capillary electrophoresis was performed using an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Europe, Zug, Switzerland) according to the manufacturer's instruction and analyzed using the GeneMapper® ID v3.2.1 software (Life Technologies Europe, Zug, Switzerland), with a minimum peak height threshold of 50 Relative Fluorescence Unit (RFU). The commercial DNA CEPH 1347-02 (Life Technologies Europe, Zug, Switzerland) was used as positive control of amplification and internal standard for allele designations. For standard PCR reactions (28-30 cycles) 0.5 ng of CEPH 1347-02 DNA was used, for all PCR reactions with increased number of cycles (34-36) 0.0125 ng of CEPH 1347-02 DNA was used.

RESULTS

Type of informative markers

As described above, the target analysis of circulating fetal DNA is based on allele-specific amplifications of those haplotypes showing DIP differences between the mother and the paternally transmitted DNA. To do so, primers are selected based on the DIP genotype of the mother. Markers informative for the genotypes of the fetus are DIP homozygous in the mother (SS or LL) and should be analyzed using primers specific to the opposite DIP allele (L- and S-primers, respectively). The fetal allele is then targeted if a DIP allele, different from the mother is transmitted by the father. Considering all possible genotype assortments, three types of informative markers exist (**Figure 1**): markers of type A, the father is homozygous for the DIP

allele that is not shared with the mother, in this case the paternal haplotype can be targeted in maternal plasma regardless which one is transmitted to the fetus; markers of type B, the father is heterozygous at the DIP locus, in this case the paternal DNA can be targeted in maternal plasma if the transmitted haplotype carries the DIP allele not shared with the mother; finally, markers of type C, the father is homozygous at the DIP locus for the same allele of the mother, in this case no paternal haplotype can be targeted in maternal plasma.

In **Supplementary Table S4** is reported the number of each type of informative marker per family. Most of the parental genotypes we studied showed two and three markers of type A, about 8 of type B and 6 of type C. Interestingly, the few parents (ID 6,15 and 18) showed no markers of type A had between five and ten choices of type B markers that allowed in practice the detection of the paternally transmitted DNA.

Detection of cffDNA by selected DIP-STRs

To test if DIP-STR markers could be used to detect fetal DNA across families and time points, each plasma sample was genotyped for at least one marker of type A. When many informative markers were available, the shortest was selected for this pilot study. As described above, families 6, 15 and 18 showed no marker of type A, therefore markers informative of the paternally transmitted alleles were identified by testing several markers of type B.

To further check for PCR specificity, we first genotyped plasma samples where the fathers were heterozygous for the STR composing the DIP-STR haplotype. The results of few representative samples are illustrated in **Figure 2**. Samples relative to family 2 never worked because of the insufficient blood collection (2.5 ml instead of 10 ml) (**Table 2**). Seven plasma samples gave positive results after modification of the PCR protocol to include 36 cycles and eight plasma samples never worked with the selected DIP-STRs. This represent 6.9 % of false negative results

considering eight fetal DNA amplifications out of 116 PCR tests performed. The markers associated to these weak and negative results are all characterized by low PCR efficiency as previously reported,[35,36] these are L-rs35032587-STR, S- and L-rs71070706-STR and L-rs111478323-STR and L-rs112604544-STR. To report on the signal-to-noise ratio of the assay, in **Supplementary Figure S1** are shown the electropherograms results of cffDNA detection in maternal blood for the samples collected during the first and second trimester that showed low RFU values (<100 RFU). Overall, some markers appeared more sensitive than others, but the quantity of the PCR product obtained did not correlate with the time in pregnancy.

Detection of cffDNA using long DIP-STRs

The use of long DIP-STR markers on a reduced number of samples also showed positive results. Alleles of 300, 305, 335, 337, 348, 360 and 386 base-pairs were successfully amplified in nine different families (ID 30and 32, 42 and 47, 48, 8, 13, 31) using different markers and samples collected at both early and late pregnancy. The results of few representative samples are illustrated in (**Figure 3a**). These families were selected solely based on the occurrence of long DIP-STR informative markers of type A, without other specific selecting criteria.

Detection of cffDNA during early pregnancy

The blood samples collected during the first trimester of pregnancy were mostly drawn between 12 and 13 weeks of gestational age. One sample (ID 49) was collected at 10 weeks and 2 days of amenorrhea and could be used to test the DIP-STR based fetal DNA detection in early pregnancy. The only marker tested with this sample, S-MID1739-STR gave a positive result (**Figure 3b**).

Correlation between fetal DNA quantity and DIP-STR results

The subset of plasma samples collected from women bearing male fetuses during the first and second trimester were selected to quantify the fetal DNA fraction and to investigate a possible correlation to DIP-STR results. The data reported in **Supplementary Table S5** indicate that positive results are obtained using as low as 0.029 ng of fetal DNA representing 3.5 % of plasma DNA. The strength of the signal (RFUs) varies depending on the sensitivity of the markers used as previously reported. Note that samples showing false negative results (NR) do not show the lowest fetal DNA quantity or fraction.

DIP-STR markers not used for cffDNA detection

Because of the specific occurrence of informative markers in these families, not all available DIP-STRs have been used for cffDNA detection. Markers MID1013–D5S490, MID1950 - D20S473, rs60194384-D15S1514, rs72534187-STR, rs138331044-STR, MID73-STR were not tested in this study. Markers MID1107-D5S1980, rs35708668-D5S2045 and rs10564579-D3S1282 were sometimes among the informative markers but they were not selected because of their long size (600-700 bp). Finally, some markers were tested for only one DIP-STR allele such as L-rs67842608-D5S468, S-rs66679498-D2S342, L-rs10564579-D3S1282, L-rs72406828-STR, L-rs145423446-STR, L-rs139592446-STR, S-rs36194161-STR, S-MID1739-STR.

DISCUSSION

Our results show that cell-free fetal DNA in maternal plasma can be detected by applying novel DIP-STR genetic markers, that we originally developed for the analysis of DNA mixtures encountered in forensic trace analysis. The results showed positive detection of fetal DNA, regardless of sex type, in the plasma of 48 women whose blood was collected during the first, second and third trimester of pregnancy. With few exceptions, all tested markers could detect the fetal DNA, including longer ones targeting DNA fragments up to 386 base-pairs. Positive results

were also obtained with one sample collected during early pregnancy at 10 weeks of gestational age. Yet, more samples collected between six and 10 weeks are necessary to validate the method for early noninvasive diagnostic purposes.

Overall, we observed a 6.9 % (8/116 PCR assays) of false negative results. The plasma samples (N=31) in which the fetal DNA fraction could be quantified by Y chromosome markers, showed values from 3.5 % to 19.2 % and no correlation between low fetal DNA quantity or fraction and DIP-STR results. No issue of fetal DNA extraction efficiency was reported by the internal control of extraction. Moreover, families with false negative results showed low marker performance longitudinally during the first, second and third trimester (see Table 2 for ID 17, 19, 21, 35, 37 and 42) and often with the same few markers, such as rs717070706-STR. Based on the observations, we interpreted the negative PCR results as mainly due to few low sensitive markers. On the other hand, no false positive result were generated by the large excess of maternal DNA, in agreement with our marker validation results that assessed allele-specificity up to a mixture ratio of 1:1000. These results confirm the sensitivity and specificity of the DIP-STRs for targeting the minor DNA component of plasma DNA, even when fetal DNA molecules are of reduced numbers, such as in early pregnancy or because of the use of long markers.

These data provide the basis for the development of a non-invasive prenatal paternity test, rapid and cost-effective, easy to implement (one or two PCR multiplex) in any forensic or medical genetics laboratory. Such molecular approach would offer several advantages with respect to currently developing methods: i) a targeted approach that guarantees a careful separation between paternity tests and disease diagnoses (aneuploidies and mutation discoveries), ii) a molecular system that is shared with other forensic and medical applications where the risk for false positive and negative result is carefully measured and the appropriate statistical framework

is available (Bayesian approach). Finally, iii) the choice of different type of informative markers (type A, B and C) provides a useful flexibility in testing different scenarios including one, multiple or unknown alleged fathers that can be associated to various degree of statistical power.

As explained in the introduction and previous publications, the number of markers capable of targeting the fetal DNA based on allele difference between maternal DNA and paternally transmitted alleles, is expected to vary across cases. As observed here with 49 families; 28 DIP-STR generate on average three markers of type A, where the parents are opposite homozygous for the DIP allele and at least one paternal allele is detected; seven marker of type B, that means one paternal allele could be detected if transmitted and seven markers of type C, that is mainly used for exclusion since negative results are expected. To bring our method closer to medical practice, we plan to, first develop the statistical framework necessary for the analysis of DIP-STR genotypes in the context of paternity testing. Bayesian models will be used as they are generally accepted in forensic science to evaluate the strength of DNA evidence. Second, based on the model above, we will study how likelihood ratios values vary depending on the number and types of informative markers considering a set of simulated families.

Our results also showed that the sensitivity of the technique may vary across DIP-STRs and we recorded four DIP-STRs (out of 19 markers tested on clinical samples) not sensitive enough for a positive/robust amplification of circulating fetal DNA in the conditions tested. Therefore, a suitably designed multiplex for amplification is needed including careful measurements of markers sensitivity and specificity, especially to assist the interpretation of results from markers of type B and C. Ideally, in case of negative results one should have a universal fetal DNA marker to demonstrate the presence of amplifiable fetal DNA. Moreover, we plan to explore the use of a larger (> 2ml) volume of plasma for DNA extractions. Note that the drawing of more

blood (> 10 ml) is possible without adducing important discomfort to the patient. Moreover, Streck BCT blood collection tubes could be used to facilitate the sample collection within a clinical environment. These tubes reduce the need for immediate plasma preparation by stabilizing nucleated blood cells at temperatures between 6°C to 37°C, for several days. Although we achieve high levels of allele-specificity with the DIP-STR genotyping approach, (markers are specific up to 1:1000 DNA ratio) and we didn't observe issue of maternal DNA amplification, it is still possible that reducing the quantity of background maternal DNA the performance of less sensitive markers improves. This strategy could be the solution for early pregnancy tests, if studies on a larger cohort indicates that marker sensitivity is limiting. However, it should be noted that several reports indicated that the quantity of cffDNA varies little during pregnancy.

Finally, of interest is an initial comparison of this method to published noninvasive prenatal paternity tests even at this early stage of development (no calculation of paternity index for the described cases) where 'inconclusive results' are due to false positive and false negative markers. Ou's group published several articles[32-34] where they use SNPs panels for the analysis of paternally inherited alleles in maternal plasma using MPS techniques. These studies show that, although results change with the specific technology used (sequencing technology, library preparation, coverage, number of SNPs), a panel of 720 SNPs has 11 % of poorly performing markers that need to be eliminated and a low signal-to-noise ratio that requires considering samples with a minimum fetal DNA fraction of 2 %. When this threshold is used and suboptimal SNPs are eliminated, 10 % to 50 % of paternally transmitted alleles from the subset of informative SNPs (about 40 % on average) are filtered out, and 0 % to 11 % when samples are collected during the second trimester. Events of allele drop-in are also observed by using MPS

with three extra alleles in 20 families reported. With respect to studies using SNP arrays,[28] the authors declare that each individual SNP measurement is noisy but technical false positive and false negative rates are not reported. Conversely, the 384 SNP method by allele-specific PCRs of Guo et al. 2012[29] indicated 13.3 % of false positive and 2.7 % of false negative rates. In our study the false negative rate is 6.9 % including poorly performing DIP-STRs (4 markers) that were not eliminated and false positive results were not observed. Indeed, interrogating a large number of markers, as for MPS or SNP arrays, offers the advantage of reducing the issue of false positive and false negative results, however an appropriate signal quality control and statistical framework for results interpretation needs to be developed.

Therefore, of interest is the possibility of integrating novel large-scale technologies although keeping a targeted approach. The use of high throughput sequencing, instead of capillary electrophoresis, for fragment analysis will offer an improved capacity in the number of markers analyzed and more flexibility in primer design. The requirement of this sophisticated technology will be elucidated after measuring the statistical power of this initial set of DIP-STR markers on data from a large number of families simulated for paternity tests.

In summary, we show that the use of DIP-STR markers for targeting the analysis of fetal DNA circulating in the blood of a pregnant woman is possible and can be used for noninvasive prenatal paternity testing. Further study is warrant to determine the number of informative markers necessary for obtaining conclusive results on paternity across parental genotypes. Conversely, for diagnostic purposes entirely new DIP-STRs should probably be developed depending on the genomic region of interest. In this regard, we previously estimated that several thousands of DIP-STRs exist[35] in the genome and that their availability should not limit the adaptation of this technique to specific needs.

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FIGURES LEGENDS

Figure 1 Types of informative DIP-STRs. Markers of type A, the mother and the father are homozygous for different DIP alleles. With this marker, the paternally transmitted DIP-STR haplotype can be targeted in maternal plasma using allele-specific primers. Markers of type B, the mother is DIP homozygous and the father is DIP heterozygous. With this marker, the paternally transmitted DIP-STR haplotype can be targeted in maternal plasma only if the transmitted haplotype carries a DIP allele not shared with the mother. Markers of type C, the mother and the father are homozygous for the same DIP allele. With this marker, no paternally transmitted DIP-STR haplotype should be detected in maternal plasma.

Figure 2 Examples of fetal DNA detection in maternal plasma with DIP-STR markers. Electropherogram results of the positive detection of the paternally transmitted alleles in maternal blood collected during the first and second or third trimester. The detected allele can be compared to the paternal genotype indicated below. The STR heterozygosity of the father allows to confirm the target amplification of the paternal halotype transmitted to the fetus. Primers are selected to be specific to the paternally transmitted alleles of the cffDNA, therefore as expected no PCR product is detected when using the reference DNA of the mother (**a**): family 51, marker rs36194161-STR. (**b**): family 31, marker MID473-STR. (**c**): family 27, marker MID473-STR.

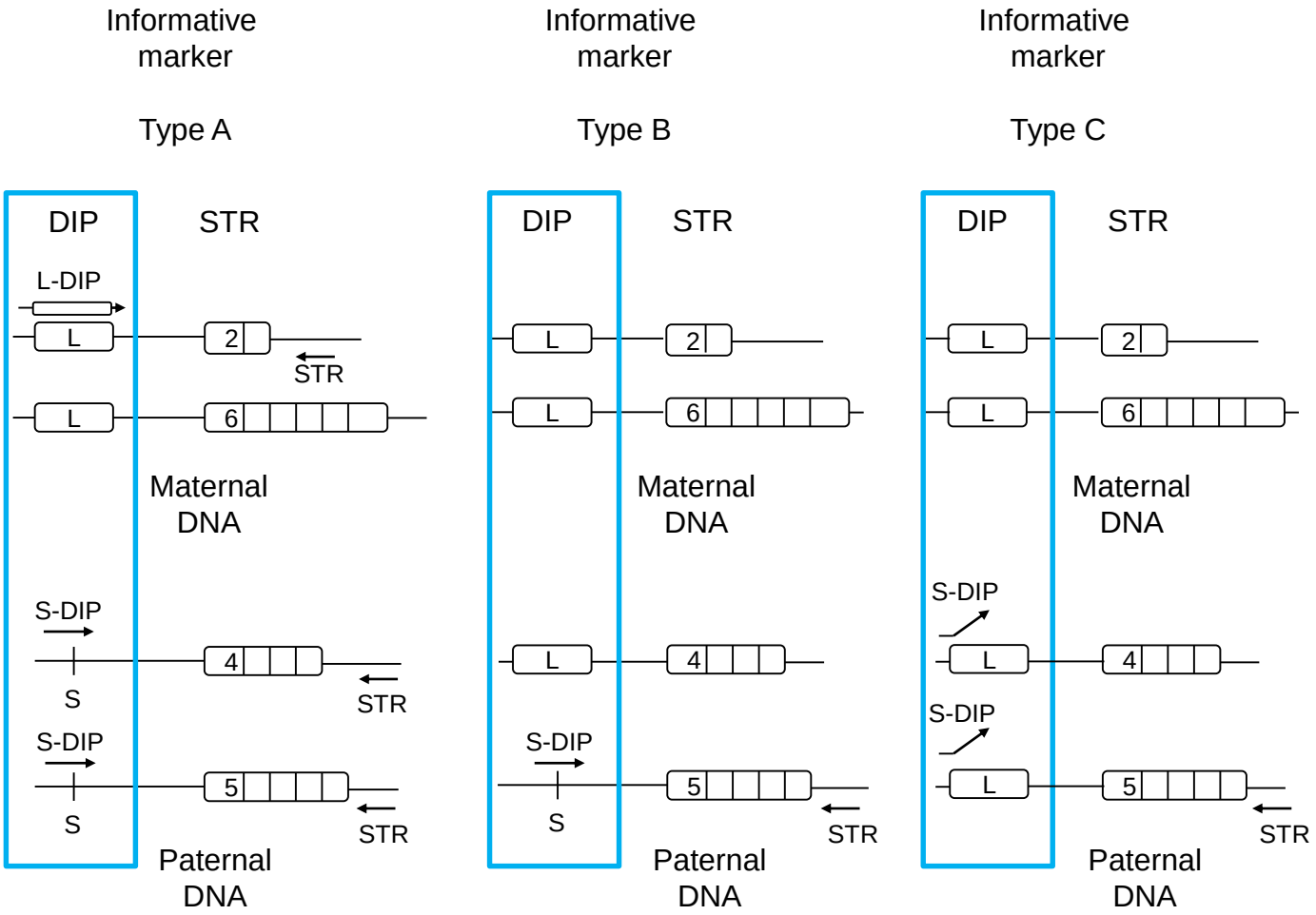
Figure 3 Examples of fetal DNA detection in maternal plasma with long DIP-STR markers and one example of fetal DNA detection at ten weeks of pregnancy.
Electropherogram results of the positive detection of the paternally transmitted alleles in maternal blood using long DIP-STR markers. (**a**): Above, family 13, marker L-rs11277790-D10S530. Below, family 31, marker L-rs67842608-D5S468. (**b**): Electropherogram results

showing one case of positive detection of the paternally transmitted allele in maternal plasma collected at ten weeks of pregnancy. Family 49, marker S-MID1739-STR.

CONFLICT OF INTEREST NOTIFICATION PAGE

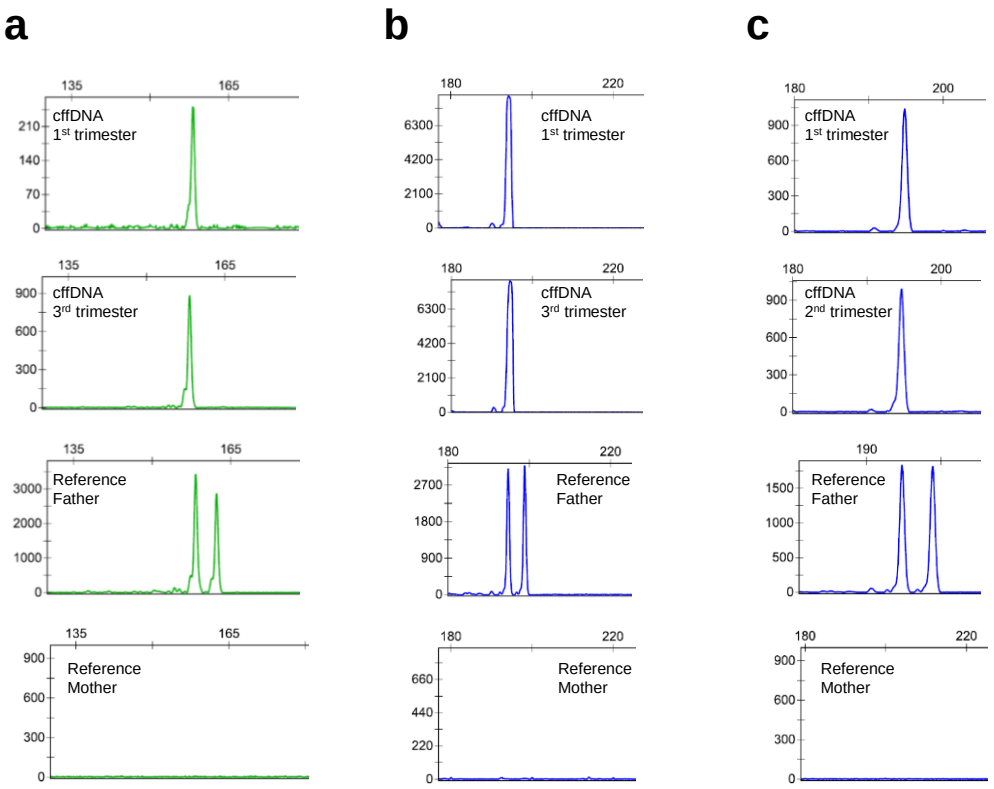
The authors declare no conflict of interest.

Figure 1


Informative marker
Type A
DIP
STR
L-DIP
L
2
STR
L
6
Maternal DNA
S-DIP
S
4
STR
S-DIP
S
5
STR
Paternal DNA
Informative marker
Type B
DIP
STR
L
2
L
6
Maternal DNA
L
4
S-DIP
S
5
STR
Paternal DNA
Informative marker
Type C
DIP
STR
L
2
L
6
Maternal DNA
S-DIP
L
4
S-DIP
L
5
STR
Paternal DNA

Figure

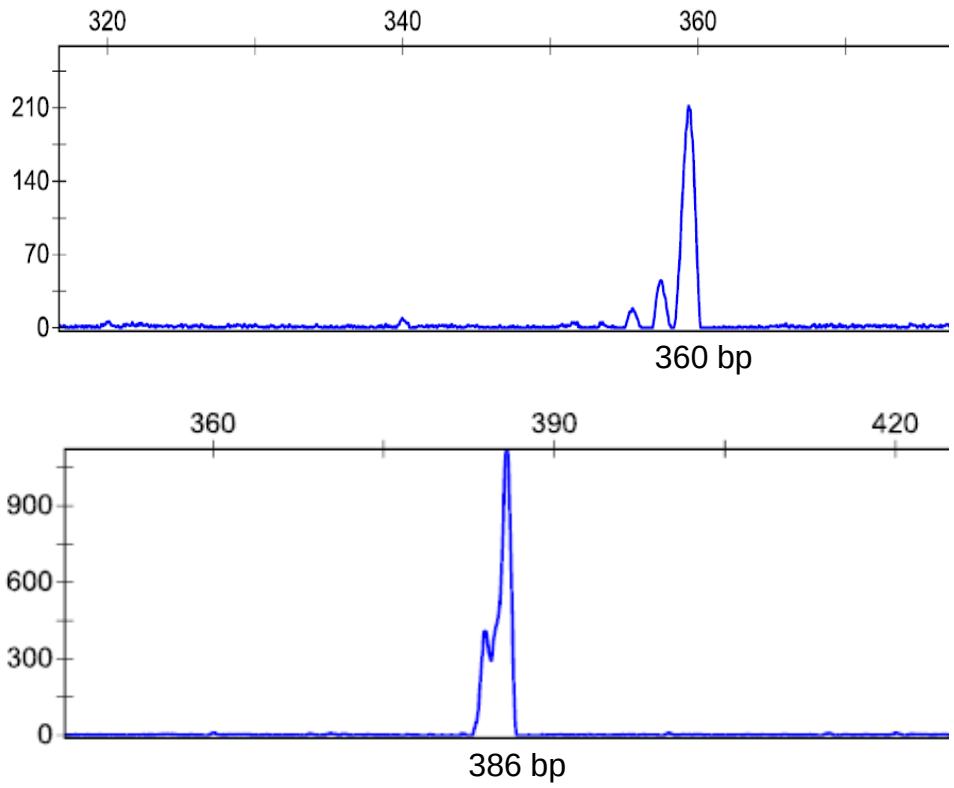
Figure 2



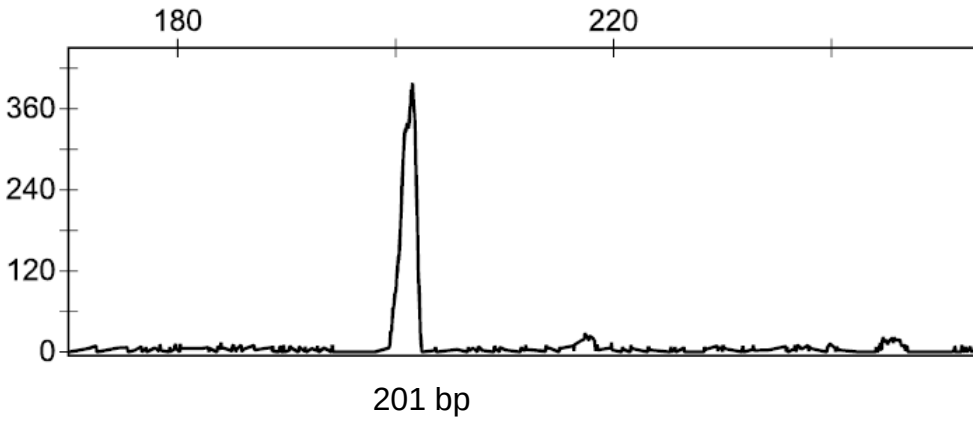
Figure

Figure 3

a



b



Table

Table 1 Plasma samples and relative gestational age of the fetus

Mother ID	First trimester	Second trimester		Third trimester	
1		24.3		37.0	
2		15.0	26.4	36.0	
3				34.1	38.1
4				28.5	
6				27.6	39.0
7		16.5		30.4	35.5
8	12.5			27.6	39.4
9		16.2		36.1	
10	12.0	25.1			
11				32.1	37.4
12	13.2	22.2		35.5	
13		23.2		36.2	
14		15.5		35.6	
15	12.2	22.1		36.6	
16				34.0	
17	12.2	21.1		37.1	
18	13.5			32.1	
19	13.0	24.2		38.3	
20	12.4				
21	12.3	25.4		39.0	
22	12.4			33.5	
23	12.6			37.5	
24		16.0	26.3		
25		15.6	24.5	36.5	
26		16.1		28.0	37.2
27	13.0	22.6			
28	13.3			36.3	
29	12.3			29.5	36.6
30	13.0	23.0			
31	12.3	25.3		36.3	
32		16.5		29.5	37.3
33	12.6			34.2	
34		15.2		28.0	36.6
35		16.0		32.3	
36	13.0	25.1			
37		16.3		30.5	35.6
38		16.0			
41		20.5		35.5	
42	11.6			27.3	
43	12.0				
44	12.5	24.4		37.5	
45	12.2	25.1		36.2	
46	12.6			29.3	39.5
47	12.4			33.1	39.2
48	13.0			37.0	
49	10.2			30.0	36.0
50		16.5	26.3	35.5	
51	12.4			34.6	

Samples 5, 39 and 40 were not included because of study participation withdrawal.
Gestational age is indicated in weeks.days.

Table

Table 2 Marker size and peak height in Relative Fluorescence Unit (RFU) of fetal DNA specific amplifications from maternal plasma

Mother ID	Marker	Size (bp)	First trimester (RFU)	Second trimester (RFU)		Third trimester (RFU)	
1	rs35032587-STR-L	259		53[a]		50[a]	
2	rs35032587-STR-S	247-251		NR	NR	NR	
3	MID1739-STR-S	210				2766	600
4	rs139592446-STR-L	159				8780	
6	rs34212659-STR-S	191				297	99
7	rs142543564-STR-L	234		54[a]		64[a]	NT
8	MID2824-STR-S	198	50			4881	4865
	rs11277790-D10S530-S	348	70			NT	NT
9	MID2824-STR-S	198		8590		420	
10	MID2824-STR-S	198	58	1288			
11	rs142543564-STR-S	210				412	52
12	rs71725104-STR-S	219	546	172		1051	
13	rs145423446-STR-L	244		1718		5031	
	rs11277790-D10S530-L	360		210			
14	rs36194161-STR-S	170		51		121	
15	rs34212659-STR-S	191	4109	2160		1175	
16	rs146332920-STR-L	179				651	
17	rs71070706-STR-S	241	NR	NR		60	
18	rs146332920-STR-S	192	688			923	
19	rs111478323-STR-L	240	93[a]	NR		192	
20	MID2824-STR-S	198	57				
21	rs71070706-STR-L	241	NR	NR		211	
22	MID473-STR-S	195	86			1871	
23	MID1739-STR-S	210	1898			5646	
24	rs72406828-STR-L	200		584	484		
25	rs139592446-STR-L	159		162	7000	7000	
	MID1013-D5S490-L	337		52	210	NT	
26	rs34212659-STR-S	191		1070		1763	4371
27	MID473-STR-S	195	1042	993			
28	MID2824-STR-S	198	59			1017	
29	MID473-STR-L	211	1439			825	1867
30	MID2538-STR-S	300	7000	6849			
31	MID473-STR-S	195	8169	6700		8077	
	rs67842608-D5S468-L	386	1000	NT		NT	
32	MID2538-STR-S	300		118		431	105
33	rs111478323-STR-L	240	50			102	
34	MID2824[a]-STR-S	198		567		9175	8340
35	rs71070706-STR-L	249		NR		67	
36	MID473-STR-L	215	8157	8070			
37	rs112604544-STR-L	160		NR		57[a]	31[a]
38	rs71725104-STR-L	227		87			
41	MID473-STR-L	215		1200		896	
42	MID2538-STR-L	305	NR			164	
43	rs111478323-STR-S	240	52				
44	MID2824-STR-L	214	55	360		673	
45	MID1739-STR-S	210	376	550		6024	
46	MID1739-STR-S	210	247			130	2715
47	MID2538-STR-L	305	1265			595	NT
	rs36194161-STR-S	161	54			996	
	rs34212659-STR-L	187	68			551	
48	rs66679498-D2S342-S	335	128			336	
49	MID1739-STR-S	201	359			146	398
50	rs72406828-STR-L	210		1606	1300	392	
51	rs36194161-STR-S	158	252			884	

[a] 36 cycles of PCR.

NT Sample not tested.

NR Sample showing negative results.

Supplementary Table S1 DIP-STR marker list

DIP-STR	Chr.	DIP S/L sequence	STR repeat	DIP-STR size[b] (bases)	Reference
MID1013-D5S490	5q23.2	-/CCAG	GT	299-345	Castella et al. 2013
MID1950 -D20S473	20p13	-/ATT	TTA	205-238	Castella et al. 2013
MID1107-D5S1980	5p15.33	-/AACA	CA	650-680	Castella et al. 2013
rs11277790-D10S530	10q25.1	-/TCCAACT	GT	340-371	Castella et al. 2013
rs60194384-D15S1514	15q26.2	-/TCTTAA	TATC	283-325	Castella et al. 2013
rs67842608-D5S468	5q11.2	-/TGGTTTAA	GT	379-395	Castella et al. 2013
rs66679498-D2S342	2q32.3	-/CCAACTTTCTCCTAC	CA	331-359	Castella et al. 2013
rs10564579-D3S1282	3p24.1	-/GTCATA	CA	714-728	Castella et al. 2013
rs35708668-D5S2045	5q34	-/TACTATGTAC	CA	621-649	Castella et al. 2013
rs35032587-STR	15q26.1	-/TATT	AGAT	239-271	Oldoni et al. 2015
rs142543564-STR	2q34	-/TACT	ATAA	210-238	Oldoni et al. 2015
rs34212659-STR	7p14.1	-/AGG	TGAA	182-199	Oldoni et al. 2015
rs112604544-STR	1q25.3	-/TTTAA	TTCC	134-204	Oldoni et al. 2015
rs111478323-STR	2p25.3	-/GAGA	TTTA	229-265	Oldoni et al. 2015
rs146332920-STR	9q31.3	-/AGG	TAAA	179-207	Oldoni et al. 2015
rs71070706-STR	1p12	-/TGT	AAAG	212-264	Oldoni et al. 2015
rs72406828-STR	4q21.3	-/ATTG	AATTT	178-250	Oldoni et al. 2015
rs145423446-STR	16p13.2	-/AGTC	GATA	230-256	Oldoni et al. 2015
rs71725104-STR	13q31.3	-/ATAG	AAAT	211-235	
rs72534187-STR	5p13.1	-/ACAGGCC	ATAG	208-236	
rs139592446-STR	2q24.2	-/ACTTAGTC	CATC	154-174	
rs36194161-STR	2q32.1	-/CTC	TTTA	138-178	
rs138331044-STR	1p12	-/CATATGC	AGAT	266-302	
MID473[a]-STR	6q16.1	-/TTACATTT	AGGA	179-227	
MID2538[a]-STR	15q25.3	-/TGTT	AC	299-311	
MID1739[a]-STR	6q14.1	-/GTCAGG	TG	201-226	
MID2824[a]-STR	11p13	-/AGGACT	AAAC	197-222	
MID73[a]-STR	22q12.3	-/GAA	CCACT	362-442	

[a]Marker name is from the Marshfield database and corresponds to rs140762, rs3054057, rs397772033, rs11278940, rs16365, respectively.

[b]DIP-STR sizes are based on the genotypes of the HGDP-CEPH global reference population (1064 individuals), except for markers MID1107-D5S1980, rs67842608-D5S468, rs10564579-D3S1282, rs35708668-D5S2045 that were genotyped in a reduced sample of 103 Swiss individuals, as described in Castella et al. 2013.

Supplementary Table S2 DIP-STR primer sequences (5' - 3') for the subset of unpublished markers

Marker	DIP primer	STR primer	S-DIP allele-specific primer L-DIP allele-specific primer DIP-STR reverse primer
rs71725104-STR	*TTTTTGCCACAAAACTAAATT GCAGCTCCTGCAAAAATTTC	*GTGTGGTGGTAGCTGGGACT TTTTGTGGCAAAAACTTTGG	CCTTTCCTTCTATTCTTGCTTTAT.TTT CCTTTCCTTCTATTCTTGCTTTATCTA *GTGTGGTGGTAGCTGGGACT
rs72534187-STR	*GCTCATGCAATTGATCAAACC GCTTTGGGCTTGATACAGAAA	*GGTATGCAATCTATCCTGATGTGA TGCATGAGCCAATTTATCTGA	TCTCTGGTTCTCTAGCTTGTAGAT.TAC CTCTAGCTTGTAGATGGCCTGTTA *AGGCCAAAATTGACATTATAGTTTA
rs139592446-STR	*ACTGGGAAAACTATGAGAAACAAA CCTTCCTTTTATCTTCTATCACACA	*GATTTAGGAGGGGATGTGGT TTTCCCAGTGTCTGCTCAA	CCATTTTGCCCCACTAGT.TC TGCCCCACTAGTGACTAAGTTC *TAGCCTTCTGCCCAAACATC
rs36194161-STR	*CCAAGATTGTGCCACTGC TCACCAGGTCTTGGGTATGTT	*AAAACATACCCAAGACCTGGTG TTTAAACCTCTTTCCTGCTTGC	AAATATTACTAGTTGTATTAGTCTGTT.ACG TATTACTAGTTGTATTAGTCTGTTCTCAC *TGCAGTGAGCAGGGTGAC
rs138331044-STR	*ACAATCGCTGCTCACTGAAG GCCGAAGCAGGTGTATTTCT	*AGCACATAGCAGGCACTAGC GCGATTGTGCCACTACACAG	ATTAGCTGGGCTTAGTG.CCTGT TAGCTGGGCTTAGTGGCATATG *GCACTAGCTGTTAGTTCCTTTTCTG
MID473-STR	*AAATGTTAAGCCTCCCCTGTC CCACTGACAGCAACAACCAA	*CCTTGTCTTGGTTGTTGCTG TGCAGGCAGATTTTAAAGGAA	TGGGCTTTCTA.TTACATTTTTAGC TGGGCTTTCTATTACATTTTTACAT *TGCAGGCAGATTTTAAAGGAA
MID2538-STR	*AACAATCTTGGCACCCATTT GCTCGCAAAGTAGGCAAGTT	*TCATTACCTTCTCTGCATTGGA GTGCCAAGATTGTTGGTGTG	GTTCAAAATCACAATCACTCA.TTT TCAAAATCACAATCACTCAAACA *TGGAATCACTCATTACCTTCTCTG
MID1739-STR	*GCACACATGTAGCAATGGTTT GCTCGCTCTTAATTGCATCC	*GGGAATTCATCCTCATTTTCC AACCATTGCTACATGTGTGCTT	CGTAGATATGTGTCTCTA.CCTGACTG ATATGTGTCTCTACCTGACCCTGAC *GGGAATTCATCCTCATTTTCC
MID2824-STR	*TGTTCCACTTCTGCCATGTG TCTAGTGGGGGTTTGCAGAG	*ACTTGGGAGGCTGAGACAGA CACATGGCAGAAGTGGAACA	TCCAAGATGAGCACTG.GGC CCTCCAAGATGAGCACTGAGTC *CCAGCCTGGCAACAGAGTA
MID73-STR	*TGTGTTTCTAAGGAGCGCTGT CACAGTGAGGAGAAGGAAGGA	*CCATTCTCTCCTTCCTTCTCC CCTGGTGCCAGAGCACAT	CATACTCAGAACTGCCTT.GAAAAG CATACTCAGAACTGCCTTGAAGAA *GCACATGGCTCTTTAATACACTG

*Fluorescent labeled primers. In the last column dots indicate the insertion/deletion point and underlined is the inserted sequence. Primers for markers previously published were not changed.

Supplementary Table S3 DIP-multiplex reactions

Multiplex group	Marker	Primers DIP (nM)
1-annealing temperature 52°C, 28 cycles	MID1013-D5S490	100
	MID1950 -D20S473	200
	MID1107-D5S1980	100
	rs11277790-D10S530	100
	rs60194384-D15S1514	200
	rs67842608-D5S468	100
	rs66679498-D2S342	200
	rs10564579-D3S1282	200
	rs35708668-D5S2045	100
2-annealing temperature 55°C, 34 cycles	rs35032587-STR	200
	rs142543564-STR	100
	rs34212659-STR	100
	rs112604544-STR	100
	rs111478323-STR	50
	rs146332920-STR	50
	rs71070706-STR	50
	rs72406828-STR	150
	rs145423446-STR	100
3-annealing temperature 55°C, 34 cycles	rs71725104-STR	150
	rs72534187-STR	75
	rs139592446-STR	150
	rs36194161-STR	150
	rs138331044-STR	120
4-annealing temperature 55°C, 30 cycles	MID473-STR	100
	MID2538-STR	100
	MID1739-STR	100
	MID2824-STR	400
	MID73-STR	400

Supplementary Table S4 Number of informative markers

Mother ID	N informative markers		
	type A	type B	type C
1	2	1	11
2	1	3	9
3	4	8	3
4	6	8	6
6	0	10	12
7	3	8	10
8	6	6	13
9	5	6	7
10	9	6	4
11	2	9	5
12	5	4	3
13	3	3	5
14	4	9	6
15	0	5	6
16	7	5	6
17	1	10	9
18	0	10	6
19	3	10	5
20	4	7	3
21	2	2	8
22	2	9	4
23	5	8	6
24	2	7	11
25	3	12	5
26	3	5	7
27	3	8	10
28	3	9	9
29	3	7	7
30	2	8	6
31	2	9	5
32	4	3	6
33	2	8	7
34	2	6	10
35	1	12	8
36	2	4	9
37	3	8	9
38	3	8	6
41	2	8	9
42	2	5	9
43	3	11	6
44	1	6	12
45	2	9	6
46	3	9	6
47	2	6	9
48	3	8	6
49	4	11	7
50	4	8	9
51	4	7	6

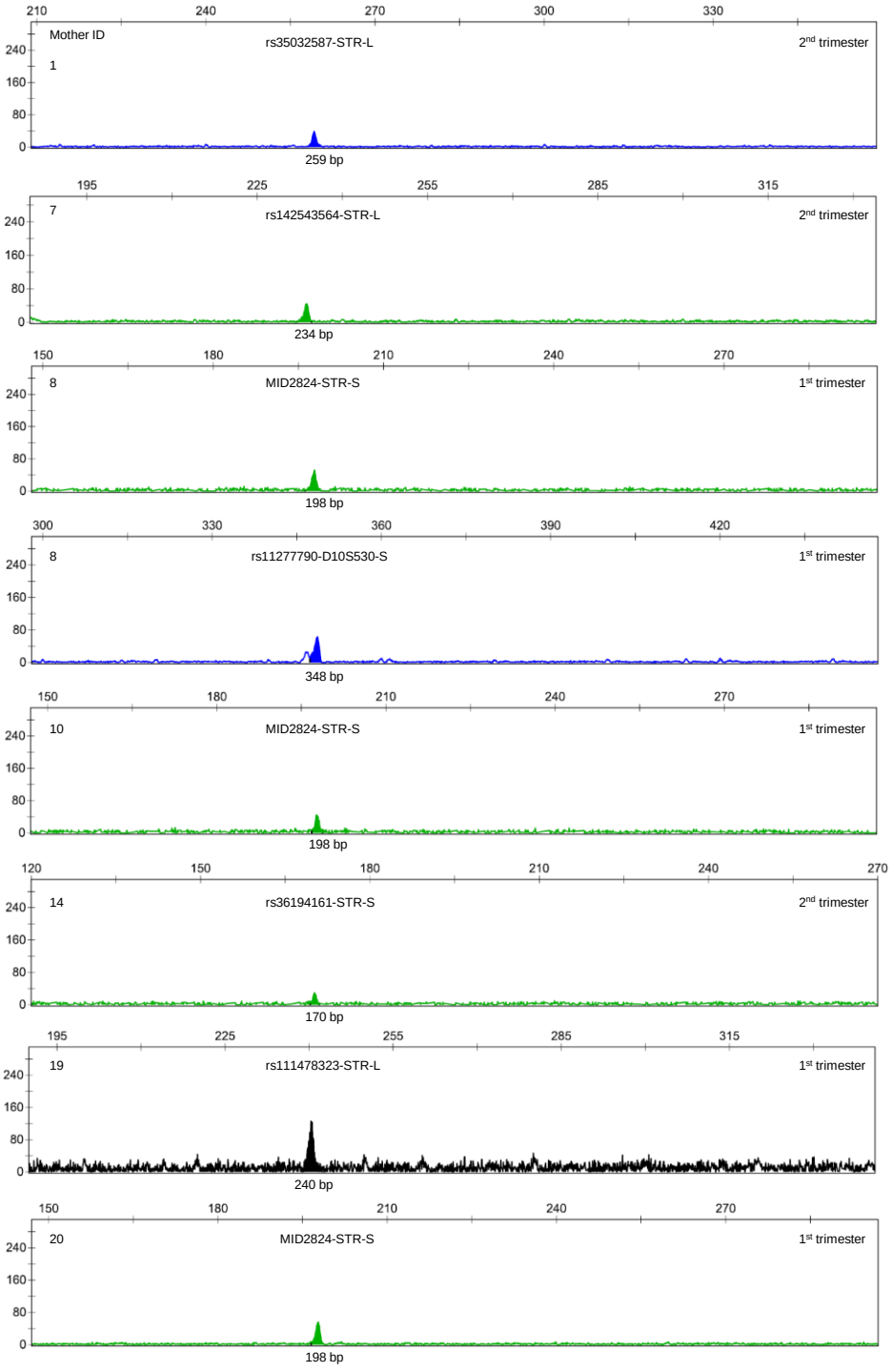
See Figure 1 for description of informative markers.

Supplementary Table S5 Quantity and percent of fetal DNA in the subset of pregnant women bearing male fetuses with samples collected during the 1st and 2nd trimester

Mother ID	Trimester	Quantity of fetal DNA amplified (ng)	Percent of fetal DNA in plasma cfDNA	Results of Table 2 (RFU)
7	1st	0.035	4.4	54
8	1st	0.056	5.5	50
9	1st	0.035	9.4	8590
12	1st	0.074	8.0	546
12	2nd	0.140	15.7	172
14	1st	0.066	6.3	51
19	1st	0.084	15.1	93
19	2nd	0.107	15.2	NR
21	1st	0.058	14.4	NR
21	2nd	0.157	17.2	NR
22	1st	0.089	6.4	86
24	1st	0.067	9.7	584
24	2nd	0.044	10.3	484
26	1st	0.841	10.3	1070
30	1st	0.117	7.6	7000
30	2nd	0.080	6.8	6849
32	1st	0.072	6.3	118
33	1st	0.072	6.1	50
34	1st	0.080	9.2	567
35	1st	0.072	8.7	NR
36	1st	0.051	4.6	8157
36	2nd	0.063	10.0	8070
38	1st	0.034	7.1	87
41	1st	0.029	3.5	1200
42	1st	0.024	5.7	NR
43	1st	0.025	10.5	52
47	1st	0.036	6.6	1265
48	1st	0.035	10.1	128
49	1st	0.029	7.8	359
50	1st	0.071	11.8	1606
50	2nd	0.112	19.2	1300

NR Sample showing negative results.

Figure S1



Electropherograms results of the subset of cffDNA detections that showed low RFU values (<100 RFU) during the first and second trimester. For sample 47, rs3619461-STR-S rs34212659-STR-L markers were amplified in multiplex.

Figure S1 (continued)