Y-chromosome identification in circulating cell-free fetal DNA using surface plasmon resonance

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What's already known about this topic? Currently, the most frequent technique to perform Y choromosome identification using circulating cell free fetal circulating DNA obtained from maternal peripheral blood is the quantitative real-time PCR.
What does this study add? Circulating cell free fetal DNA obtained at early gestational ages and not detectable by conventional quantitative real-time PCR, can be discriminated with high accuracy and reliability using SPR-based biosensors.

ABSTRACT

Objective Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma, diagnostic non-invasive prenatal methods have been developed or optimized for fetal sex determination and identification of genetic diseases. As far as fetal sex determination, this might be important for therapeutic intervention on sex-associated pathologies such as Duchenne muscular dystrophy, hemophilia, congenital adrenal hyperplasia. Surface-plasmon resonance (SPR)-based biosensors might be useful for these studies, since they allow to monitor the molecular interactions in real-time providing qualitative and quantitative information, through kinetics, affinity and concentration analyses.

Methods The Biacore X100 has been applied to identify the Y-chromosome in cffDNA obtained from plasma samples of 26 pregnant women at different gestational ages. We have performed SPR-based analysis of SRY-PCR products using SRY-specific probes immobilized on the sensor chip.

Results We have demonstrated that there is a statistically significant difference between samples collected by pregnancies carrying male or female fetuses. Moreover, cffDNA obtained at early gestational ages and not detectable by conventional quantitative real-time PCR, can be discriminated with high accuracy and reliability using SPR-based biosensors.

Conclusions These data, in addition to their direct applicability in more extensive diagnostic trials, should be considered as the basis of future developments.

1 **INTRODUCTION**

2

3 The presence of circulating cell-free fetal DNA (cffDNA) in plasma or serum of healthy
4 pregnant women has been reported,[1,2] putatively originating from trophoblast breakdown.[3]
5 cffDNA comprises only a small portion of total cell-free DNA. The earliest studies suggested
6 that the fetal fraction was only 3-6% of total DNA in maternal plasma,[4] but more recent
7 studies have found that it may be closer to 10-20% in the last weeks of gestation.[5,6] It is
8 possible to detect circulating cffDNA from 4 or 5 weeks of gestation[7] until delivery. The
9 gestational age positively correlates with amount of fetal DNA in plasma. While the quite
10 stable cffDNA in maternal circulation is highly fragmented, the entire fetal genome is fully
11 represented.[8,9] As far the size is concerned, it was found that the 85.5% of fetal DNA is shorter
12 than 0.3 kb in early pregnancy (13 or 15 weeks of gestation),[10] and constitutes the 28.4% of
13 the < 0.3 kb fraction in maternal plasma, increasing to 68,7% in the third trimester.[10]
14 Since the discovery of cffDNA in maternal plasma,[2] non-invasive prenatal sampling methods
15 have been applied and extraction and analysis techniques have been developed or optimized
16 for the isolation and detection of fetal DNA with a diagnostic aim.[12] In particular, fetal gender
17 determination,[13] fetal rhesus D genotyping,[14] pregnancy-associated conditions,[15]
18 aneuploidies,[11] monogenic disorders have been investigated.[16] As far as fetal gender
19 determination, this is extremely important in those cases where the mother is carrier of an X-
20 linked disorder, such as Duchenne muscular dystrophy or hemophilia,[17,18] because
21 pregnancies with male fetuses are primarily at risk, or for those at risk of conditions
22 associated with ambiguous development of external genitalia, for example congenital adrenal
23 hyperplasia, where early maternal treatment with dexamethasone can reduce the degree of
24 virilization of female fetuses.[19,20]
25 Traditionally, early fetal gender determination has been performed using invasive techniques,
26 such as chorionic villus sampling or amniocentesis. These procedures, however, still carry a 1-
27 2% risk of miscarriage and cannot be performed until 11 weeks of gestation.[21,22] Therefore
28 prenatal diagnostic procedures without risk for the fetus and based on the analysis of
29 circulating fetal genetic material in maternal blood have been developed.[23,24]
30 The molecular determination of fetal gender is based on the recognition of Y-chromosome-
31 specific sequences in maternal blood using nested-PCR, quantitative real-time PCR, digital
32 PCR.[25,26] The commonly utilized loci for Y-chromosome-specific sequences, amplified with a
33 quantitative real-time PCR (qRT-PCR) are the single copy SRY gene,[4] the multicopy DYS14
34 within the *TSPY* gene,[2,17] and the multicopy DAZ gene,[27] but at early gestation, it is quite

1 difficult to detect the very low amount of circulating cffDNA.[4,28,29] In addition, in non-invasive
2 prenatal diagnosis, the identification of female fetuses employing Y-chromosome-specific
3 sequences is based on a null result, but this may be the source of false negative results if the
4 amount of fetal male DNA is so little that it cannot to be detected by qRT-PCR. Moreover, this
5 method could lead to false positive due to possible male DNA contamination of the sample
6 during the extraction of DNA from maternal plasma. Therefore, other approaches would be
7 required for detecting also fetal female DNA.
8 Surface plasmon resonance (SPR) based biosensors, such as Biacore X100, allow to monitor in
9 real-time the interactions between biomolecules. After the ligand immobilization on the
10 sensor chip surface and the subsequent injection of the analyte, their possible interaction
11 produce an increment in mass resulting in a change of the SPR angle, which is monitored in
12 real-time as resonance signal in function of time in a sensorgram.[30] SPR biosensors have been
13 used to study a wide range of biomolecular interactions, providing both qualitative and
14 quantitative information through by kinetic, affinity and concentration analysis.[31,32]
15 This innovative technology has been applied in many different fields, such as the
16 measurement of glucose levels in the blood,[33] the search of genetic modified organisms
17 (GMOs) in food,[34] the diagnosis of point mutations causing diseases, such as β thalassemia and
18 cystic fibrosis,[35,36] the detection of pathogens, toxins, veterinary compounds and chemical
19 additives in food specimens.[31]
20 In this study the possibility of detecting sequences of the Y chromosome in pregnant women
21 was evaluated, with the aim of identifying the gender of fetuses by SPR-based biosensors.
22 Peripheral blood samples were collected from 26 pregnant women at different weeks of
23 gestation and then the fetal DNA was extracted from the plasma. SPR-based analysis of SRY-
24 PCR products using a Biacore X100 instrument and SRY-specific probes immobilized on the
25 sensor chip were employed to detect the Y chromosome of the male fetuses. All these results
26 were compared with the actual gender of the newborns and data obtained by conventional
27 quantitative Real-Time PCR (qRT-PCR).
28
29 **METHODS**
30
31 **Samples collection**
32 Blood samples were collected by using test tubes containing EDTA anticoagulant. After
33 approval by the Ethical Committee of S. Anna Hospital Ferrara (Italy), about 18 ml of blood
34 were sampled from pregnant women. In all cases informed consent was obtained, and the

1 experiments were conducted in agreement with the Declaration of Helsinki. A progressive
2 number was assigned to each specimen to ensure the anonymity of the donor.
3

4 **Plasma preparation**

5 Plasma was prepared within 3 hours from blood collection, according the protocol described
6 in literature.[37] Briefly, after mixing tubes in a rotator for 5-10 minutes, samples were
7 centrifuged at 1200 x g for 10 minutes at 4°C without brake. Plasma was then carefully
8 collected and centrifuged again at 2400 x g for 20 minutes at 4°C in order to completely
9 remove platelets and precipitates. The resulting supernatant was collected and stored at -
10 80°C into single-use aliquots.
11

12 **Extraction of circulating cell-free DNA**

13 DNA was extracted from 2 ml of maternal plasma, not thawed more than once, by using the
14 QIAamp® DSP Virus Spin Kit (Qiagen, Hilden, Germany), according to the manufacturer's
15 instructions. DNA elution was performed in 60 µl of AVE buffer.
16

17 **Quantitative real-time PCR (qRT-PCR)**

18 6 µl of circulating DNA extracted by maternal plasma were analyzed by using real-time PCR
19 amplification assays specific for the β globin gene (forward: 5'-GCAAAGGTGCCCTTGAGGT-3';
20 reverse: 5'-CAAGAAAGTGCTCGGTGCCT-3'; BETA PROBE: 5'-
21 FAM/TAGTGATGG/ZEN/CCTGGCTCACCTGGAC/3IABkFQ-3'), and for the SRY gene (forward:
22 5'-CCCCCTAGTACCCTGACAATGTATT-3'; reverse: 5'-TGGCGATTAAGTCAAATTCGC-3'; SRY
23 PROBE: 5'FAM/AGCAGTAGA/ZEN/GCAGTCAGGGAGGCAGA/3IABkFQ-3'), in order to quantify
24 total and fetal (in case of male fetus) DNA, respectively. Every reaction, containing TaqMan®
25 Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA), had a final volume of 15 µl
26 and was performed in duplicate, except circulating DNA samples derived from pregnant
27 women at early gestation (< 10 weeks), where four different reactions were run at the same
28 time for the SRY determination. For each analysis, some standards containing known
29 amounts of male genomic DNA were prepared, to make a calibration line for the absolute
30 quantification of samples. No-template controls were included as well. The reactions were
31 carried out on a StepOne™ Real-Time PCR System (Applied Biosystems, Life Technologies),
32 by using the StepOne Software (Applied Biosystems, Life Technologies) and the following
33 amplification program: 2 minutes at 50°C; 10 minutes at 95°C; 40 amplification cycles

comprising a denaturation step of at 95°C for 15 s and an annealing-elongation step at 60°C for 1 min.

Unbalanced polymerase chain reaction (PCR)

An unbalanced amplification was required to produce single-stranded PCR products for biospecific interaction analysis with Biacore™ X100. First, 6 µl of circulating DNA extracted from maternal plasma underwent a PCR with specific primers (SRY-SB: 5'-GAGGCGCAAGATGGCTCTAGAG-3'; SRY-SC: 5'-CCACTGGTATCCCAGCTGCTTGC-3'), amplifying a 73 bp sequence of the SRY gene, located on the Y-chromosome. PCR was performed in a final volume of 50 µl, containing 12.5 µM dNTPs, 150 ng of PCR primers and 1.25 U of ExTaq DNA polymerase (TaKaRa, Otsu, Shiga, Japan). The 50 PCR cycles included: denaturation, 30 s, 94°C; annealing, 30 s, 65°C; elongation, 10 s, 72°C. When required, the second pre-amplification of samples was performed in the same conditions, using 5 µl of the first PCR product as a template. Finally, the unbalanced amplification was carried out from 5 µl of the balanced PCR product, using the forward primer SRY-SB alone and the conditions just described. The secondary structure of the single-stranded SRY-SB PCR product was determined by using the *The mfold Web Server (http://mfold.rit.albany.edu/?q=mfold/)*.[38]

Biospecific interaction analysis with Biacore™ X100

The Biacore™ X100 analytical system (Biacore, GE Healthcare, Chalfont St Giles, UK) was used in all experiments, as well as SA sensor chips (Biacore, GE Healthcare), precoated with streptavidin, and the running buffer HEPES-buffered saline-EP+ (HBS-EP+: 0.1 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) (Biacore, GE Healthcare). The experiments were conducted at 25°C temperature and at 5 µl/min flow rate. In order to immobilize the biotinylated SRY probe (5'-biot-CTCTGAGTTTCGCAT-3') on the SA sensor chip surface, the well-documented streptavidin-biotin interaction was employed.[39] After pretreatment with three 10 µl pulses with 50mM NaOH - 1M NaCl, an injection of 40 µl of HBS-EP+ containing the oligonucleotide probe at the concentration of 10 ng/µl was administered in the flow cell 2 of the sensor chip. The analysis of biospecific interaction with target DNA was carried out by injecting 60 µl of HBS-EP+ buffer containing the unbalanced SRY PCR product, followed by a washing step with the running buffer alone. After hybridization, the sensor chip was regenerated by performing a 5 µl pulse of 50 mM NaOH. Sensorgrams were analyzed with the Biacore™ X100 Evaluation Software, version 2.0.1 (Biacore, GE Healthcare). A subtraction of the background signal recorded on the empty flow

cell 1 was automatically produced. In addition, suitable blank control injections with running buffers were performed, and the resulting sensorgrams subtracted from the experimental results. Resonance unit (RU) values were measured after both the analyte injection (RUfin) and the washing step (RUres).

Statistical analysis

All the Biacore™ data were normally distributed. Statistical differences between groups were compared using one-way ANOVA (ANalyses Of VAriance between groups) software. Statistical significance was assumed at $P < 0.05$.

RESULTS

Detection of fetal Y-chromosome sequences by real-time PCR

The most frequently employed technique to identify the fetal gender is qRT-PCR.[4] Accordingly, we employed an amplification assay specific for the SRY gene using a wide range of samples with particular attention to early gestational ages, where generally the fetal sex is still unknown.

After the extraction of circulating DNA from 2 ml of plasma specimens derived from 26 pregnant women at different gestational weeks, the detection of total DNA and cffDNA was carried out by qRT-PCR using specific amplification assays for the beta-globin[4] and the SRY genes[4] respectively. The fetal gender was considered male or female according to the generation or not of a SRY-specific amplification plot, respectively. The actual sex of the future newborn and the diagnostic outcome are reported in **Table I** for all samples, listed in a decreasing order according to the gestational week.

These results demonstrated that the formulated diagnoses were correct and a proper fetal gender diagnosis could be performed by qRT-PCR starting from the 9th gestational week. For some samples at 7th and 6th week of gestation (#22 and #25), the diagnosis was not clear, suggesting the need of alternative approaches with higher sensitivity and suitable for molecular diagnosis of monogenic diseases, such as those employing Biacore biosensors and SPR technology.[35,36]

Detection of fetal Y-chromosome sequences by Biacore™ X100

In order to perform a fetal male DNA detection in earlier gestation periods, we assayed the Biacore technique, already validated for high sensitivity and applicability to molecular diagnosis.[31,34-36, 40-45] An oligonucleotide probe recognizing the SRY gene, located on the Y chromosome, was immobilized on a sensor chip; then the hybridization with an injected unbalanced PCR product obtained by circulating DNA purified from maternal plasma was evaluated (**Figure 1A**). Unbalanced PCR product is needed to obtain efficient hybridization with the immobilized probes. In these experimental conditions, only in the case of male target DNA a hybridization signal is expected.

The target PCR product was obtained in two different steps. In the first, the SRY gene of the target DNA, if present, was amplified with the two SRY-SB and SRY-SC primers[46] (**Figure 1B**), generating a 73 bp product containing a 15 nucleotides region corresponding to the sequence of the immobilized probe. The second step consisted in a second unbalanced amplification of the first amplicon with the use of only the forward primer (SRY-SB), in order to obtain a single-stranded SRY target sequence complementary to the probe. The expected secondary structure of this single-stranded product, predicted by *The mfold Web Server* (http://mfold.rit.albany.edu/?q=mfold/)[38] showed that a major portion of the sequence complementary to the probe was expected to be available for possible hybridization with the ligand DNA (**Figure 1C**). This is a key result, since heavy secondary structure of the sequence to be analyze can deeply interfere with the probe hybridization as elsewhere reported[35].
According to the described strategy, the biotinylated probe was first immobilized on the streptavidin matrix of a SA sensor chip (**Figure S1A)**.
After the probe immobilization, we tried to validate the analytical technique with the injection of unbalanced PCR products obtained using 100 ng of control genomic DNAs, one male and one female, respectively. The reaction products of a negative control (a PCR reaction without template) were also injected to exclude possible contaminations (**Figure S1B**). As expected, only after injecting the PCR product derived from male genomic DNA a hybridization-dependent SPR curve was obtained (solid line in **Figure S1B**); in addition, the interaction between the oligonucleotide probe (ligand) and the single-stranded PCR product (analyte) was rather stable, since the difference between the RUfin (212, measured after the analyte injection) and RUres (189, measured after the washing step) was very small. On the contrary, both samples deriving from female DNA (dashed line in **Figure S1B**) and PCR negative control

(dotted line in **Figure S1B**) did not generate any signal, confirming the specificity of the detection assay for male DNA.

After demonstrating that the system was working and applicable to determination of fetal sex (because the immobilized probe only binds to male DNA), the unbalanced SRY PCR products obtained by circulating DNA extracted from maternal plasma were injecteded on the sensor chip surface.

The sensorgram of **Figure 2A** shows some representative examples of the resulting interaction SPR curves. The curve relative to the pregnant woman #7 bearing a female child represents an example of what we observed in case of injection of samples enriched of female fetal DNA. No increase of the SPR signal was obtained because, as expected, hybridization did not occur between the PCR product and the SRY-specific probe immobilized on the sensor chip. On the contrary, the four SPR curves showing a ligand-analyte interaction were obtained by all samples derived from circulating DNAs of pregnant women bearing a male child, at different gestation periods. In particular, the curve belonging to #2, #9, #18, #22 samples were obtained from pregnant women at 36th, 17th, 11th, 7th weeks of gestation. In all cases, after injection of the unbalanced PCR product, an interaction with the immobilized DNA probe was observed. The following washing with HBS-EP+ buffer did not cause a significant decrease of the signal, demonstrating the generated hybridization complexes were quite stable. After each analysis, the sensor chip was regenerated by a 1 min pulse with 50 mM NaOH, in order to remove the bound analyte and to be able to perform a new hybridization experiment (data not shown).

Considering only the hybridization curves deriving from pregnant women bearing a male, we can see some variability among samples, given by different values of recorded RU, higher for the sample #2, lower for the specimen #22. We found a certain correlation between the signal obtained, expressed by bound RU values, and the gestational period, probably due to a greater amount of fetal target sequences available for the hybridization with the probe. All the nine analyzed pregnancies with female fetuses did not produce a significant increase of the SPR signal, due to absence of hybridization with the SRY probe (**Figure 2B**). The detected final RU values were very low, in average from 0 and 14 units, corresponding to a background signal really homogeneous and independent from the gestational weeks with 4.8 mean value (**Table II** and **Figure 2B**, black triangles). On the contrary, the seventeen analyzed specimens deriving from pregnant women expecting a male, showed final RU values always higher than the previous group, corresponding to an effective interaction with the probe with 81.7 as

mean value. In this case we observed a certain degree of heterogeneity among the produced signals correlated to the gestational week (**Table II** and **Figure 2B**, white dots).

These data suggest that there is a statistically significant difference between RU values obtained by pregnancies with male fetuses and with female fetuses. Moreover samples at early gestational age, such as the #22 specimen, not detectable by qRT-PCR, were able to generate a positive SPR signal using this strategy, demonstrating that this approach, based on pre-amplification PCR products injected onto Biacore sensor chip flow cells, permitted to identify the fetal sex with high accuracy until to the 7th gestational week.

As samples at the 6th week of gestation, such as #25, were not discriminate through the same procedure, we performed the analysis using a double pre-amplification in order to increase the amount of the fetal DNA for the analysis. The results demonstrated that this second approach could solve uncertain outcomes and increase the possibility to assess fetal sex from specimens collected from pregnant women at the 6th gestational week (**Table III**).

DISCUSSION

Non-invasive prenatal diagnosis has become increasingly important, because, although it retains only a predictive/probabilistic value, it allows to study the fetal health without any risk for both fetus and mother.[47] Increased numbers of pregnant women at early stages of pregnancy are expected to undergo this prenatal screening, considering on one hand the importance of the diagnostic outcomes for certain pathologies, and on the other hand the absence of potential risks deriving from the diagnostic technology.

Non-invasive prenatal diagnosis was born with the discovery of circulating cffDNA within the maternal plasma and serum[2] and since then an increasing number of studies have been performed with the aim to develop experimental non-invasive approaches for detecting, at an early gestational age, the possible presence of aneuploidies or monogenic diseases.[5,11,12]

Our study, based on the detection of circulating fetal DNA within maternal plasma, was aimed to the final development of an experimental non-invasive method of prenatal diagnosis to determine the fetal gender. To these aims, twenty-six blood samples from pregnant women with a wide variability in terms of weeks of gestation and sex of the future unborn were collected.

Currently, the most frequently used technique to identify the fetal gender is the qRT-PCR.[4] We adopted an amplification assay specific for SRY gene, located on the Y-chromosome, allowing the detection of fetal male DNA. We assessed its applicability also to the early gestational ages,

where generally prenatal diagnosis should be required. This technique succeeded to correctly identify the presence of male fetal DNA up to 9th week of gestation correctly. However, more sensitive techniques should be required to efficiently perform non-invasive detection of fetal gender.

In order to obtain a better sensitivity in male fetal DNA identification, we assayed the surface-plasmon resonance (SPR)-based Biacore technology, already known for its low detection limit, user-friendliness, reproducibility, low costs, automation.[31] In these experiments a biotinylated oligonucleotide probe specific for the SRY gene sequence was immobilized on a streptavidin-coated sensor chip, with the aim to evaluate the possible hybridization with complementary unbalanced SRY PCR products obtained by cffDNA in maternal plasma.

The results obtained were very encouraging, because we were able to demonstrate a highly specific hybridization. In fact, when a sample derived from female fetal DNA was injected on the sensor chip, no interactions were recorded and no increase in SPR-generated resonance unit (RU) values obtained. On the contrary, clearly detectable signals were produced by the injection of analytes derived from male fetal DNA. In those cases the RU values were much higher, even though probably depending on the different amounts of fetal DNA related to the gestational age. Anyway, it should be underlined that a statistically significant difference was found among signals produced by male and female samples, so that this technique resulted suitable to detect the fetal gender.

In addition, despite the fact that the number of cases analyzed were low, our data strongly suggest that Biacore X100 allows to detect male fetal DNA even in earlier gestation periods compared to the qRT-PCR technique. For example, specimens at the 7th or 6th week of gestation, when analyzed by qRT-PCR, generated an uncertain outcome, probably due to lower fetal DNA amounts, near the detection limit of the technique (0.02 pg/μl). On the contrary, a clear increase of RU, due to the detection of fetal male DNA, was observed after injection of the unbalanced PCR products amplifying cffDNA obtained from pregnant women bearing a male fetus.

It should be pointed out, however, that in cases of very early gestation (6th gestational week), a second step of pre-amplification before performing the unbalanced PCR reaction was required, in order to increase the amount of template fetal sequences to perform the Biacore analysis.

In respect to comparison with other diagnostic tools and methods, we would like to underline that (a) SPR-based instruments are at present available exhibiting low cost in respect to real-time and digital PCR devices; (b) the sensor-chip can be re-used several times (up to 80-100

fold), therefore limiting the cost of the analysis; (c) the results of the SPR-based diagnostic procedure are obtained within few minutes; (d) full automatisation of the procedure is possible; (e) arrayed SPR methodologies are available allowing high-throughput analyses.

The data reported in this paper, in addition to their direct applicability in more extensive diagnostic trials, should be considered as the basis of future developments. For instance, in order to improve the sensitivity and the specificity, the experimental strategy here reported can include the use of peptide nucleic acid (PNA) probes, known for their higher stability, sensitivity and specificity than DNA probes in molecular hybridization with complementary sequences.[35,48,49] In addition, our results are the proof-of-principle that secondary structure of the genomic stretch containing the SRY target sequence is fully permissive to hybridization, allowing the extension of the methods to a recently described SPR-based ultrasensitive PCR-free technology. This very interesting approach allows the direct detection of unamplified genomic fragments using SPR-Imaging, PNA-based hybridization and signal enhancements with gold microspheres.[49,50]

As far as other biomedical applications, this technology should be verified when non-invasive prenatal diagnosis is employed for detection of monogenic diseases in the carried fetus. While SPR-based molecules detection of point mutations has been formally demonstrated in thalassemia and cystic fibrosis,[35,42,51-53] the feasibility of the strategy described in this paper for non-invasive diagnosis of thalassemia, cystic fibrosis and other diseases in currently under investigation. Interestingly we were able to perform non invasive prenatal diagnosis using blood samples obtained from pregnant women with a fetus carrying the β-thalassemia IVSI-110 mutation (Breveglieri et al., manuscript in preparation).

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1 **FIGURE LEGENDS**

2

3 **Figure 1.** (**A**) Representative scheme of the immobilization of a SRY DNA probe on a sensor
4 chip and injection of unbalanced PCR products generated by the template fetal DNA. (**B**) Map
5 of SRY gene, reporting the sequences of SRY-SB and SRY-SC PCR primers generating a 73 bp
6 amplicon to be used for the unbalanced amplification with the forward primer alone. The
7 sequence of the antisense SRY DNA probe immobilized on the sensor chip is indicated as well.
8 (**C**) Secondary structure of the unbalanced SRY-SB PCR product, calculated by *The mfold Web*
9 *Server (http://mfold.rit.albany.edu/?q=mfold/)*[38] injected as analyte on the sensor chip
10 surface. The region complementary to the immobilized SRY probe is indicated in dark.

11

12 **Figure 2.** (**A**) Sensorgrams obtained by injection on immobilized SRY probe of unbalanced
13 SRY-SB PCR products obtained by different samples of circulating DNA extracted from plasma
14 of pregnant women at different gestation ages: #7, bearing a female fetus; #2, #9, #18, #22 all
15 bearing male fetuses. *a*, sample injection for 12 minutes; *b*, washing step with HBS-EP+ buffer.
16 RUfin (final resonance units) and RUres (residual resonance units) measured after the sample
17 injection and the washing step, respectively. The assays were performed by using the
18 Biacore™ X100 instrument, at 25°C and 5 μl/min flow rate; the running buffer was HBS-EP+;
19 the results were analyzed by the Biacore™ X100 Evaluation Software: the resulting plots
20 were obtained after subtracting the sensorgrams produced by analyte injection onto an empty
21 flow cell and by the running buffer injection. (**B**) Distribution of RUfin values obtained by
22 samples derived from male (white dots) or female (black triangles) fetuses and reported in
23 Table II. The black horizontal bars indicate the mean values for each population. *p< 0,001

24

25 **SUPPLEMETARY MATERIALS**

26

27 **Figure S1**. (**A**) Sensorgram obtained by the immobilizaton of the biotinylated SRY probe on a
28 streptavidin-coated (SA) sensor chip. Final resonance units (RUfin) and residual resonance
29 units (Rures) were measured after the sample injection and the washing step respectively. *a*,
30 sample injection; *b*, washing step with HBS-EP+ buffer. As expected, the final (RUfin) and
31 residual resonance unit values (RUres), measured after the injection and at the end of
32 subsequent washing, do not differ, confirming that the interaction between the polymer
33 matrix and the oligonucleotide is very stable.

(**B**) Sensorgram generated by the injection on the immobilized probe of unbalanced SRY-SB PCR products obtained by control male (whole line), female (dashed line) genomic DNAs and PCR negative control (dotted line). The assays were performed by using the Biacore™ X100 instrument, at 25°C and 5 µl/min flow rate; the running buffer was HBS-EP+; the results were analyzed by the Biacore™ X100 Evaluation Software. The resulting plots were obtained after subtracting the sensorgrams produced by analyte injection onto an empty flow cell and by the running buffer injection.