Facilitating implementation of NIPD: a modified protocol to detect the universal fetal DNA marker RASSF1A

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Background

- Circulating cell free fetal DNA (cffDNA) in maternal plasma is used for the non invasive prenatal diagnosis (NIPD) of fetal sex, RHD status in D- mothers and some single gene disorders
- Failure to amplify a target sequence can be interpreted as a true negative result but could also indicate absence of cffDNA in the sample
- The promoter of RASSF1A has been reported as a universal fetal DNA marker which can be used to confirm the presence of cffDNA as it is hypermethylated in placenta but hypomethylated in maternal blood
- Using methlyation-sensitive restriction enzyme digestion hypomethyated sequences can be digested leaving only hypermethylated (fetal specific) sequences detectable (Figure 1)
- To eliminate false positive results, it is important to ensure that complete digestion of maternal hypomethlyated sequences has taken place
- We have developed a modified real-time PCR protocol for the detection of RASSF1A for use as a universal fetal marker in non-invasive prenatal diagnosis

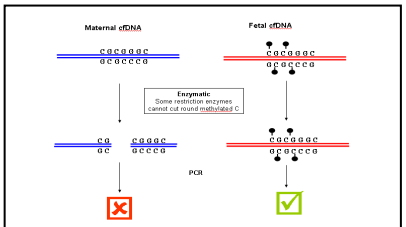


Figure 1: The RASSF1A promoter is methylated in fetal cfDNA & unmethylated in maternal cfDNA. Following restriction digest with methylation specific enzymes the maternal DNA is digested and cannot be amplified by PCR. The fetal cfDNA remains undigested and can be amplified by PCR.

Methods and Results

- Using combinations of methylation-sensitive enzymes and a methyation-insensitive digest to control for complete digestion we have tested maternal plasma samples from pregnant women (n=66) for the presence of cffDNA (Figure 2).
- Cell free DNA was extracted from 1.2 - 2ml maternal plasma using the QIAamp circulating nucleic acid kit (QIAGEN) or Qiagen EZ1 DSP Virus Kit
- Samples were analysed in triplicate with no digestion, a methylation-sensitive digest and a methylation insensitive-digest (Figure 3) and then subjected to real-time PCR analysis of RASSF1A (Figure 4)
- RASSF1A was detectable in all undigested samples (100%) demonstrating that total cell free DNA had been extracted successfully
- For the methlyation-sensitive digest, 5 (7.5%) samples had no detectable hypermethylated RASSF1A (i.e. no cf fetal DNA) and 61 (92%) had one or more replicates positive for RASSF1A indicating the presence of cf fetal DNA
- For the methylation-insensitive digest, 61 samples (92%) were negative for RASSF1A indicating complete restriction digestion and 5 samples (7.5%) had 1 replicate positive for RASSF1A indicating that restriction digestion was incomplete
- Examples of using RASSF1A as a universal marker in a NIPD test for determination of fetal sex are shown in Figure 5 (a-c)

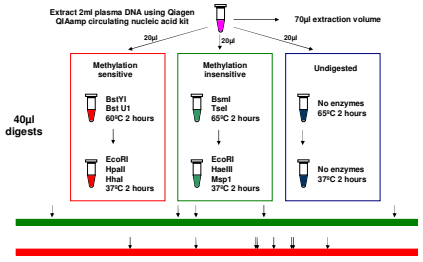
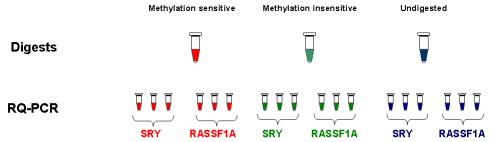


Figure 2: Cell free DNA was extracted from 2ml maternal plasma and was digested with methylation sensitive and methylation insensitive restriction enzymes. The methylation sensitive enzymes digest maternally derived DNA only. The methylation insensitive enzymes digest all DNA and act as a restriction digestion control. The undigested control allows total DNA concentration to be determined & confirms that cf DNA (maternal and/or fetal) has been extracted.



Male fetus	☑	☑	☑	☒	☑	☑
Female fetus	☒	☑	☒	☒	☒	☑
No fetal DNA	☒	☒	☒	☒	☒	☑
No cell free DNA	☒	☒	☒	☒	☒	☒

Figure 3: Schematic representation of the possible results obtained using the RASSF1A as a universal fetal marker when determining fetal sex by NIPD. Green ticks represent the presence of amplified products from either an SRY assay or the RASSF1A assay. Red crosses represent no amplification of SRY or RASSF1A amplicons.

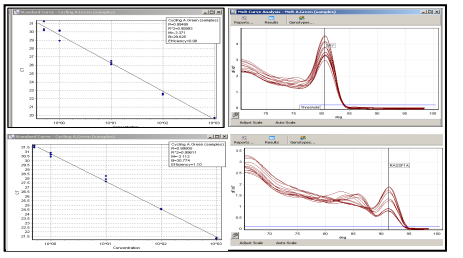


Figure 4: Examples of standard curves and melt profiles for the SYBR green real time PCR assays for SRY (top panel) and RASSF1A (bottom panel). PCR amplicons are generated using standard cycling conditions in the presence of SYBR green. Both assays show good efficiency. Following amplification the amplicons are melted to determine specificity. SRY amplicons melt at 80.5ºC and RASSF1A amplicons melt at 91.2ºC. Non specific products will have different melting temperatures.

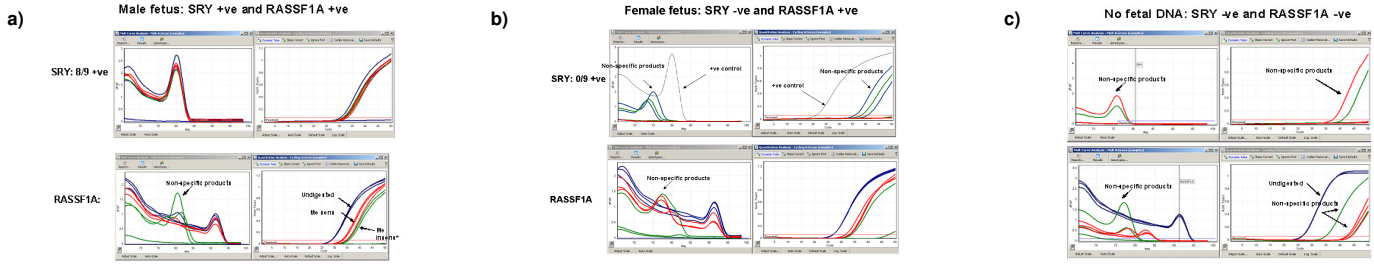


Figure 5: Example PCR amplification plots and melting profiles for the SRY and RASSF1A real time SYBR green PCR assays. a) Male fetus: 8/9 amplification replicates for SRY were positive and melted at 80.5ºC indicating the presence of SRY. RASSF1A amplicons that melted at 91.2ºC were detected in the methylation sensitive digest (red line) indicating the presence of fetal DNA and undigested control (blue line) indicating that total cell free DNA has been extracted. The methylation insensitive digest failed to amplify RASSF1A products melting at 91.2ºC indicating complete digestion of cell free DNA. b) Female fetus. No SRY amplicons that melted at 80.5ºC were observed indicating absence of SRY. RASSF1A amplicons that melted at 91.2ºC were detected in the methylation sensitive digest (red line) indicating the presence of fetal DNA and in the undigested control (blue line) indicating that total cell free DNA has been extracted. The methylation insensitive digest (green lines) failed to amplify RASSF1A products melting at 91.2ºC indicating complete digestion of cell free DNA. The fetus can be reported as female as no SRY was detected and the presence of fetal DNA has been confirmed. C) No fetal DNA extracted. No SRY amplicons that melted at 80.5ºC were observed indicating absence of SRY. RASSF1A amplifications that melted at 91.2ºC were detected in the undigested control (blue line) indicating that total cell free DNA has been extracted. However, no signal was detected in the methylation sensitive digest (red line) indicating the absence of fetal DNA. The methylation insensitive digest (green lines) failed to amplify RASSF1A products melting at 91.2ºC indicating complete digestion of cell free DNA. In this case the sample would fail analysis and a repeat sample should be requested. Without information from the RASSF1A assay this sample could have been reported incorrectly as a female fetus.

Conclusions

- This modified real time PCR assay for RASSF1A could be useful for laboratories undertaking NIPD of fetal sex determination and single gene disorders for use as a universal fetal DNA marker

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