

# Non-invasive prenatal detection of a micro syndrome mutation in a maternal blood sample using SNaPshot™ mini-sequencing

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## Introduction

- The identification of cell-free fetal DNA in maternal plasma has offered potential as a non-invasive source of fetal genetic material for prenatal diagnosis.
- Current testing focuses on fetal sexing for pregnancies at risk of X-linked disorders and congenital adrenal hyperplasia, and fetal RhD status determination.
- Application of non-invasive prenatal diagnosis (NIPD) for single gene disorders is more technically challenging due to the low concentration of fetal DNA and a predominance of very similar maternal DNA sequences.
- Current NIPD is therefore limited to the detection or exclusion of genetic sequences that are not present in the mother i.e. paternally inherited or arising de novo. This allows for modification of risk calculations in pregnancies at risk of single gene disorders and reduces invasive testing on fetuses that are not at risk.

**Aim:** To investigate a method of cfDNA analysis for the detection of paternally inherited or de novo alleles. The method was required to be highly sensitive, suitable for use with small PCR fragments, easy to optimise, robust, easy to perform and cost efficient.

## SNaPshot™ mini-sequencing

- Bustamante-Aragones *et al.* (2008) have demonstrated the use of a mini-sequencing technique called SNaPshot™ (Applied Biosystems) for the detection of paternal cystic fibrosis mutations in maternal plasma.
- The SNaPshot™ multiplex kit (Figure 1) is designed to interrogate up to ten single nucleotide polymorphisms (SNPs) at known locations on one to ten DNA templates in a single tube.
- Primers are designed to bind to a complementary template, one base upstream from the SNP site of interest, in the presence of fluorescently labelled dideoxynucleotide Triphosphates (ddNTPs) and DNA polymerase. The polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3' end.
- Capillary electrophoresis is used to identify the nucleotide present at the mutation site (Table 1).
- Initial PCR fragment size can be designed to be very small, therefore particularly appropriate for cfDNA analysis.

## Micro syndrome

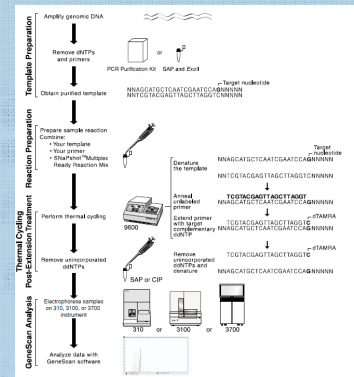
- Rare autosomal recessive disorder characterised by ocular and neurodevelopmental defects and microgenitalia.
- Associated with several mutations in RAB3GAP gene at 2q21.3.
- Sequence analysis of DNA from a chorionic villus sample received at the laboratory showed a compound heterozygote fetus with the nonsense mutations c.691C>T (p.Arg231X) and c.1039C>T (p.Arg347X). These pathogenic mutations were previously detected in the father and mother respectively.
- Maternal plasma with the paternal specific mutation was available from the laboratory's ethically approved research bank (REC10/H1207/16), therefore this made it a good candidate disorder for SNaPshot™ proof of principle experiments.

## Methods

- 2 ml of maternal plasma was used for DNA extraction using the Qiagen circulating nucleic acid kit and eluted into a final volume of 35 ul. Paternal DNA was used as a heterozygous control (positive control) and maternal DNA was used as the wild-type control for the paternal mutation (negative control). A non-pregnant plasma control was also set up.
- Several different volumes of plasma DNA (1-10 ul) and 50 ng of DNA from the controls were used as templates for PCR.
- The reaction volume was 25 ul, containing 12.5 ul of 2 x Qiagen multiplex mastermix and a final concentration of 0.2 uM for each primer (forward: 5'-ctagGGATGTCCTTTAACTCCATT-3', reverse: 5'-ATACTGCTGCCAATCTTGAAGT-3'; Invitrogen).
- After an initial incubation at 95°C for 10 min, the reaction was cycled for 1 min at 95°C, 1 min at 62°C, and 1 min at 72°C for 40 cycles, followed by a final extension of 10 min at 72°C. An amplicon of 82 bp was produced.
- 3 ul of purified PCR product was used as a template for mini-sequencing. The reaction was carried out in a final volume of 20 ul, containing 10 ul of SNaPshot™ multiplex ready reaction mix (Applied Biosystems) and a final concentration of 0.5uM for the unlabelled sequencing primer (5'-TGCCTCCAGTTAGTATTGCTATT-3'; Invitrogen).
- The reaction was cycled for 10 s at 96°C, 5s at 50°C, and 30 s at 60°C for 30 cycles.
- Excess nucleotides were removed by the addition of 2 ul (1 U/ul) shrimp alkaline phosphate enzyme and 2.4 ul of 10x SAP buffer (USB), and incubating the mixture at 37°C for 1 h followed by 75°C for 15 min.
- Products generated from the mini-sequencing reaction were run on the ABI3130 genetic analyser (Applied Biosystems) using GeneScan® analysis software to identify the nucleotide present at the mutation site.

**Table 1: Fluorophore of each ddNTP**

ddNTP	Dye label	Colour of analysed data
A	dR6G	Green
C	dTAMRA™	Black
G	dR110	Blue
T (U)	dROX™	Red



**Figure 1: SNaPshot mini-sequencing**

## Results

- SNaPshot™ mini-sequencing retrospectively detected the paternal micro syndrome mutation in the maternal plasma (Figure 2). In a diagnostic setting this would indicate that the fetus was at risk of being affected and invasive testing would be required to determine if the fetus also carried the maternal mutation.

## Discussion

- Current prenatal diagnosis involves an invasive procedure with associated risk of miscarriage.
- SNaPshot™ mini-sequencing has been shown to be a sensitive and accurate method for detection/exclusion of paternally inherited fetal mutations in maternal plasma.
- This allows for modification of risk calculations in pregnancies at risk of single gene disorders e.g. for recessive conditions where the parents carry different mutations.
- Its ease of use, rapid protocol and low cost make SNaPshot a good candidate for future routine clinical use in NIPD.

## Future work

- Need to validate the technique for other mutations.
- Fetal specific marker required to prevent false-negative results - RASSF1A assay is currently being developed in house.

## References

- A. Bustamante-Aragones *et al.*, J. Cystic Fibrosis (2008) 505-510.
- ABI PRISM® SNaPshot™ Multiplex Kit Protocol (Applied Biosystems).



**Figure 2: SNaPshot results**