Salisbury NHS
NHS Foundation Trust

Non – invasive Prenatal Diagnosis

Technical Challenges and Advances

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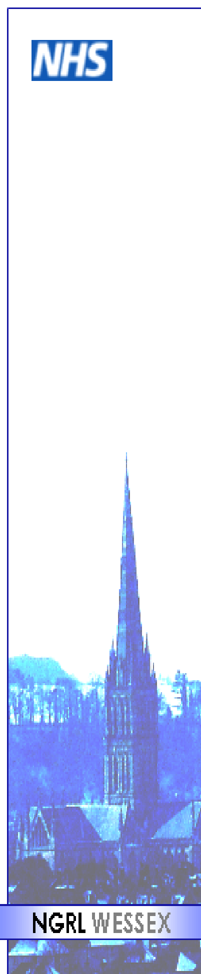
Technical Challenges for NIPD



DNA from the fetus can be detected in the blood (plasma) of pregnant women and in principle can be used to test for:

fetal sex

RhD status

inheritance of paternal mutations

Aneuploidy e.g. Down syndrome

But…

Amount of circulating fetal DNA is low – how can we extract enough DNA to perform reliable tests?

How do we know we are testing fetal DNA rather than the mother's DNA?

How can we determine copy number changes fetal DNA in a background of maternal DNA to test for aneuploidy?

Major Areas

- Sample collection and processing
- Extraction of cell free (cf) fetal DNA / (RNA)
- Fetal sexing and detection of paternal mutations
- Fetal specific markers
- Detection of aneuploidy

Sample collection and processing

Factors that affect subsequent analysis of cf fetal DNA (RNA):

- Sample transit time to lab – ideally <48 hours

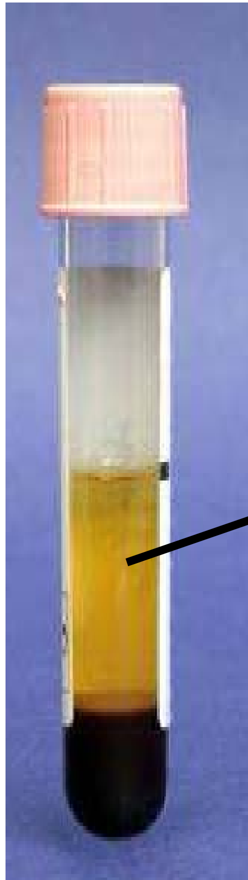
    Minimise potential blood cell lysis

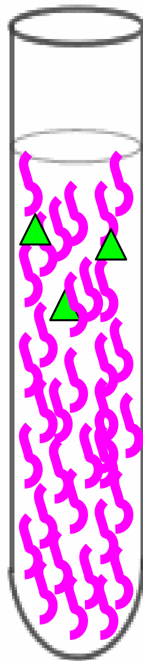
- Plasma preparation

    Different protocols have been shown affect fetal DNA quantification

    Goal is to maintain integrity of sample – keep the physical properties of the maternal and fetal derived DNA intact and ensure that plasma sample is cell free

Extraction of cell free fetal nucleic acids





11 – 17 weeks

Cell free fetal DNA (3.4%)

Cell free maternal DNA (96.6%)

Amount of cf fetal DNA extracted is equivalent to 25 genomes / ml plasma

Challenges for sample processing and cf fetal DNA extraction

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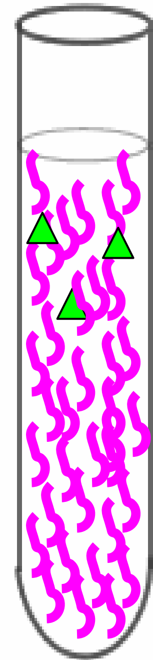
- Population variation
- Low copy number of cf fetal DNA
- Background 'contamination' with cf maternal DNA (94 – 97%)
- Challenge is to increase cf fetal DNA recovery and enrich the amount of cf fetal DNA in the total plasma DNA recovered



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Advances in cf DNA Extraction and Enrichment

1) Size fractionation

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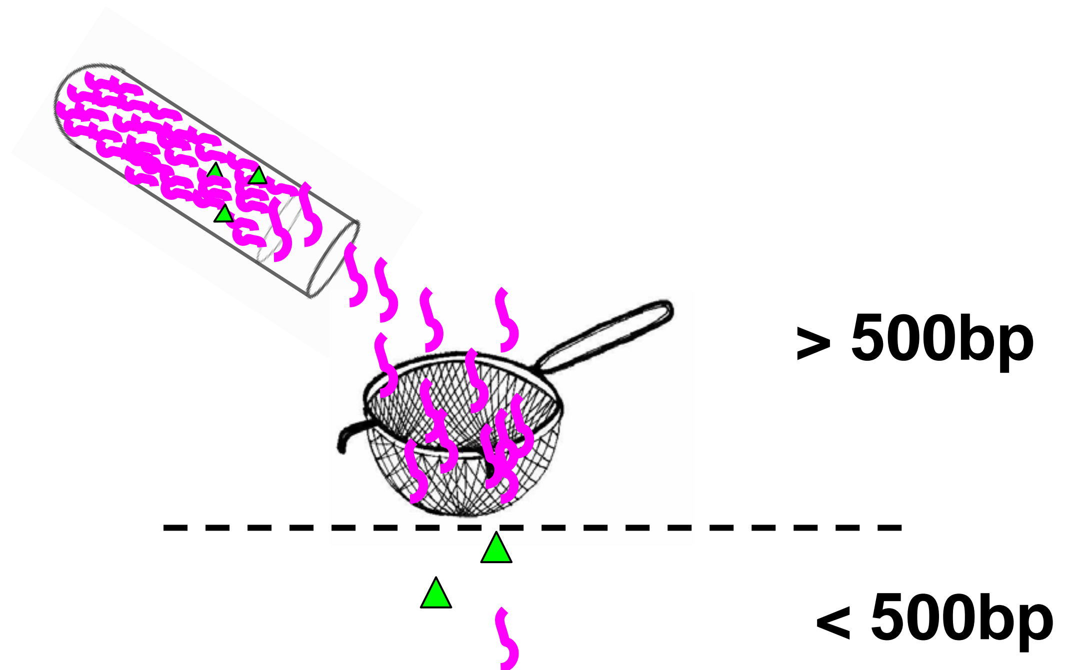
- cf fetal DNA shorter than cf maternal DNA
- 99% cf fetal DNA < 313bp in length
- Majority c.145bp or less
- cf maternal DNA significantly longer >500bp



Advances in cf DNA Extraction and Enrichment

2) Structural differences in cf fetal DNA and cf maternal DNA

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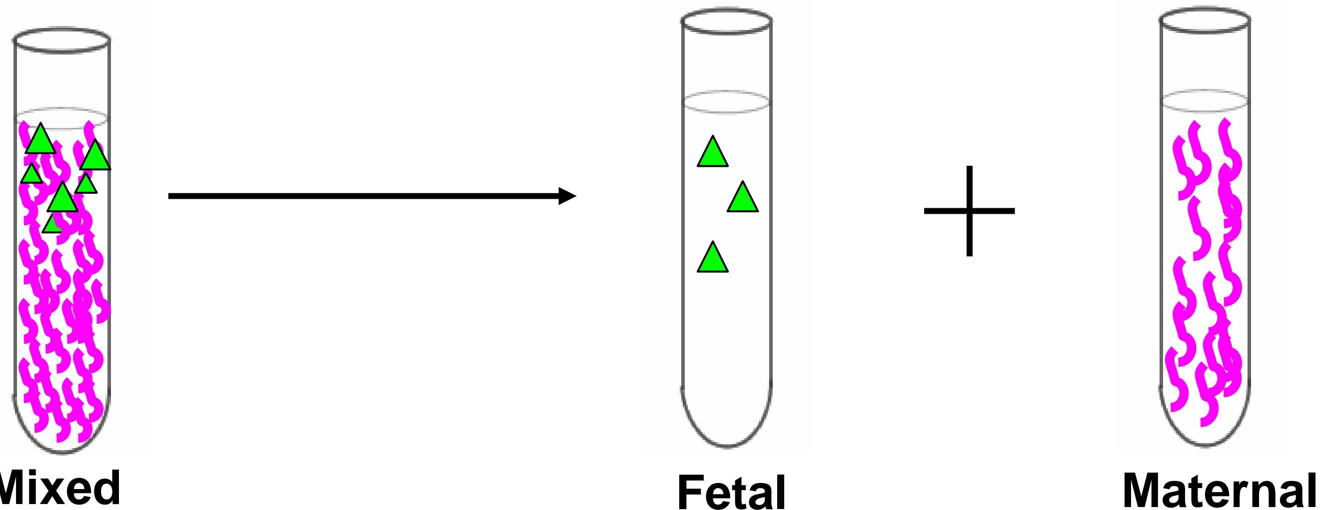
"It is believed that circulating fetal DNA is predominantly associated with nucleosomes and has a molecular structure distinct from maternal DNA.

As a result, such distinctions can be used to achieve isolation and/or enrichment of fetal DNA from maternal plasma, which will then provide an invaluable source of fetal genetic material for non-invasive prenatal diagnosis" .

Patent application #20070243549 Bischoff



Fetal sexing and detection of paternally inherited mutations

Technical challenges

- **Low copy number of cf fetal DNA (early gestational age)**

  Techniques need to have very sensitive levels of detection

- **Background 'contamination' with cf maternal DNA**

  Need to have a high enough proportion of fetal DNA present to avoid false negative results

- **Confirmation that fetal DNA is present in sample**

  Necessary to avoid false negative results

Established Techniques

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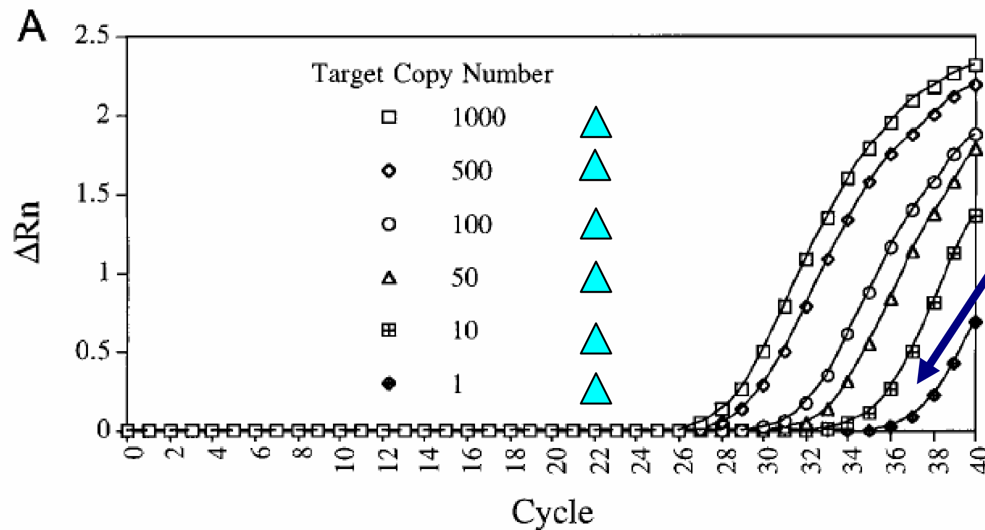
- **Real time quantitative PCR (RQ-PCR)**

Detection level c. 1 copy ~ 6.6pg DNA



Working near to limits of detection (c. 2GE/PCR) – require stringent criteria for calling of true positive and true negative results – multiple replicates, repeat extractions

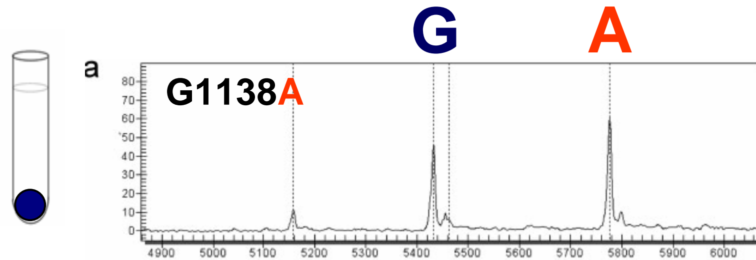
Testing for the presence detection of Y chromosome specific sequences **approaching 100% sensitivity and specificity** – multiple studies. Reliable from 7+ weeks gestation (PROOF audit and IBGRL – diagnostic implementation)

Established Techniques

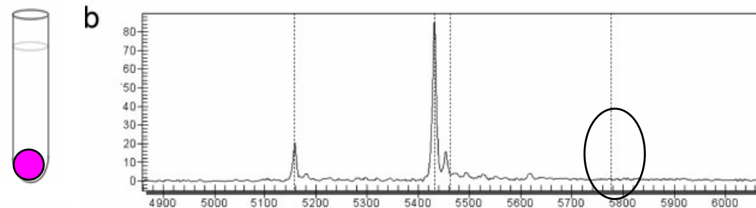
• **Matrix assisted laser desorption / ionisation time of flight mass spectrometry (MALDI-TOF)**

e.g. detection of achondroplasia: G1138A mutation in *FGFR3*

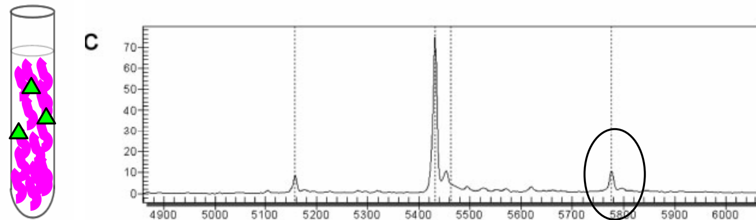
G **A**



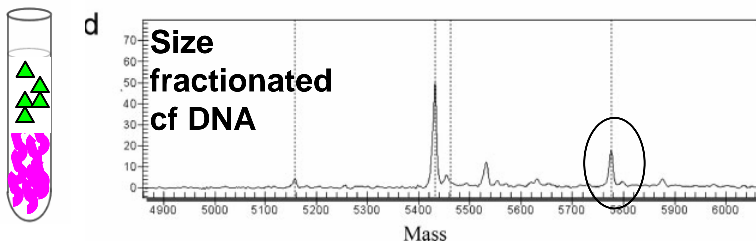
Paternal genomic DNA (G1138A)



Maternal genomic DNA (G1138)



Maternal plasma cf DNA (G1138A)



Maternal plasma cf DNA (G1138A)

Diagnostic Utility of MALDI-TOF

- **Fetal Sexing** (n=97) (Li *et al*., Clin Chem 2006)

  Sensitivity 95%

  Specificity 100%

Sensitivity = true pos / (true pos + false neg)
Specificity = true neg / (true neg + false pos)

- **Publications showing successful detection of**

  Paternal mutations e.g.Achondroplasia (Li *et al*., Pre Diag 2007)

  Paternal polymorphisms (e.g. Chow *et al* Clin Chem 2007; Li *et al* Electrophoresis 2006)

  Epigenetic markers (e.g. Chan *et al*. & Tong *et al* Clin Chem 2006)

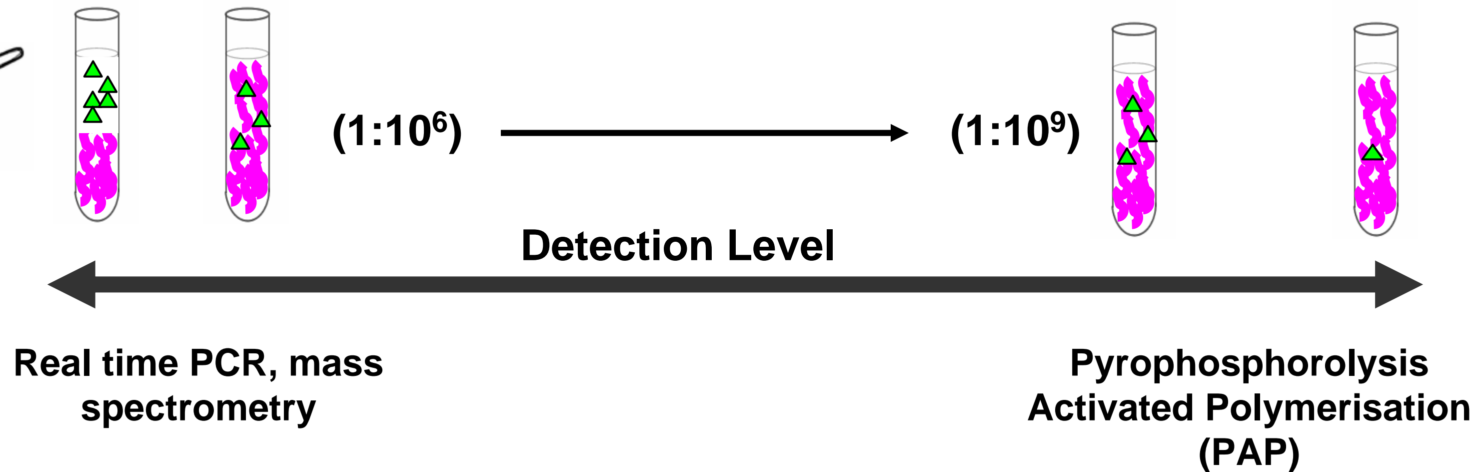
- **Sequenom Inc**

  Hold intellectual property covering all NIPD which utilises cf DNA and RNA in maternal plasma (applies to all technologies)

New advances

Development of techniques with improved detection levels

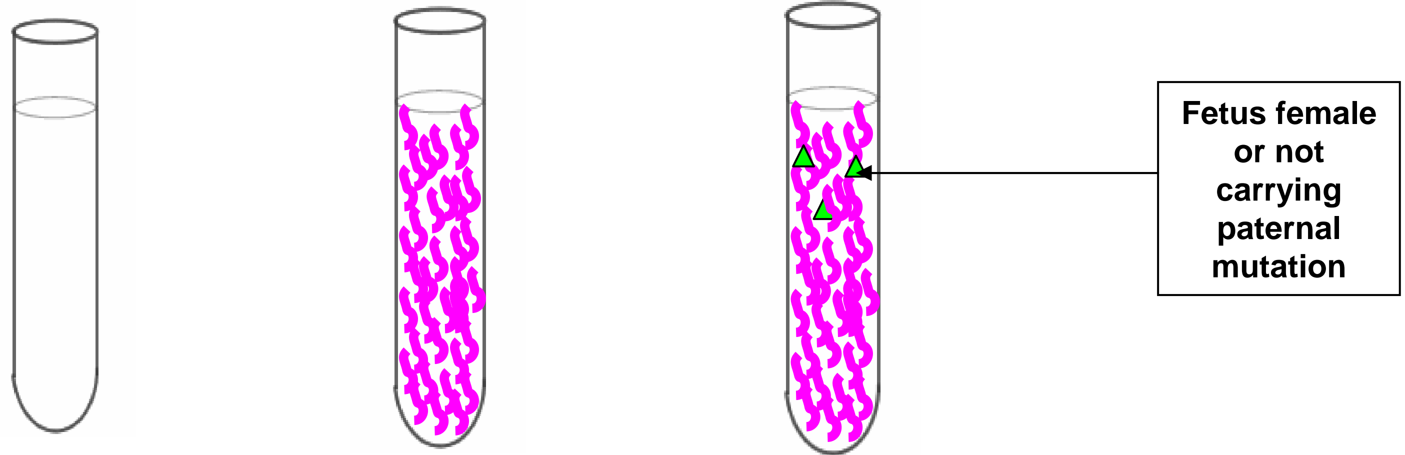
e.g.Pyrophosphorolysis Activated Polymerisation (PAP)



- Theoretical detection level of 10^{-9} (improvement on RQ-PCR)
- Fetal sexing n=54 (Boon et al., Prenatal Diagnosis 2007)
- Sensitivity 100 % (95% CI: <5.56% chance of false negative)
- Specificity 98.1 % (95% CI: 91.5% - 99.9%) [1 false positive]
- Potential for detection of paternal mutations
- Requires high purity blocked oligonucleotides – risk of false positives

Fetal specific markers

- **To confirm presence of cf fetal DNA if testing result is negative**



- **Several candidates**

Control Genes (limited) – will determine that cf DNA extracted but not distinguish fetal and maternal

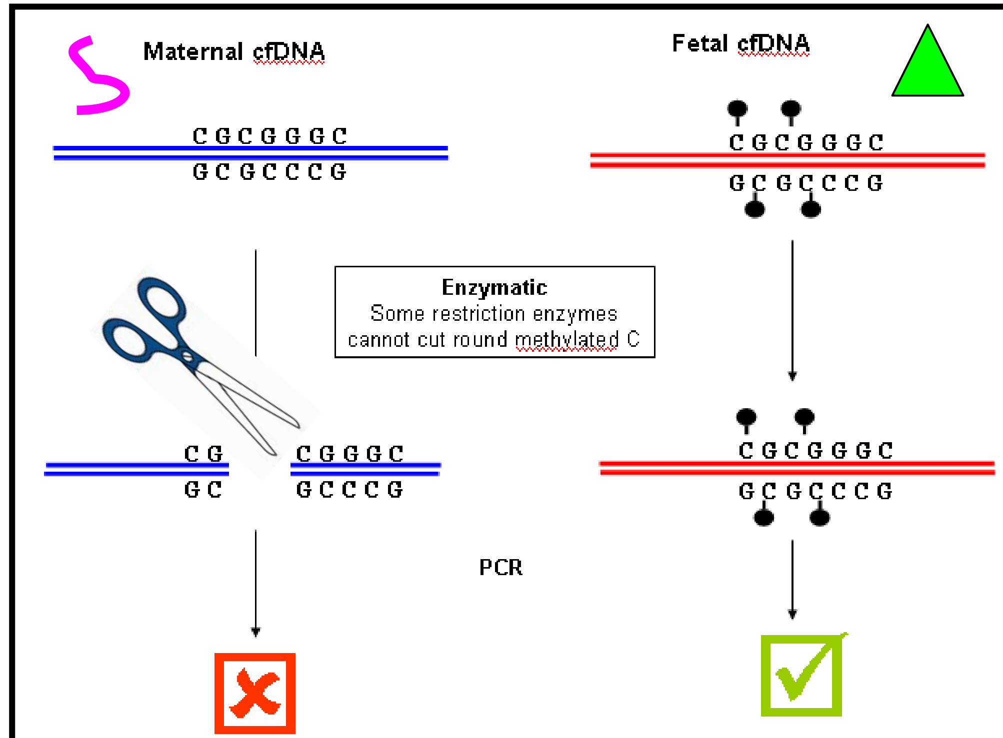
Paternal single nucleotide polymorphisms

Epigenetic markers

No one clear approach at present – many candidate markers under development

Epigenetic markers

Exploits genetic variation between mother and fetus related to methylation status of DNA



These markers are sex and polymorphism independent and therefore can be used for any pregnancy

Examples: RASSF1A (fetal hypermethylation) Chan et al., Clin Chem, 2007

SERPINB5 (fetal hypomethylation) Tong et al., Clin Chem, 2007

Detection of aneuploidy

The ability to use NIPD to detect fetal aneuploidies, particularly trisomy 21, represents a major breakthrough in prenatal diagnosis

- **Major technical challenge**

Background of cf maternal DNA mean direct quantification of fetal chromosome copy number is not yet feasible

Need targets that are free from maternal background interference

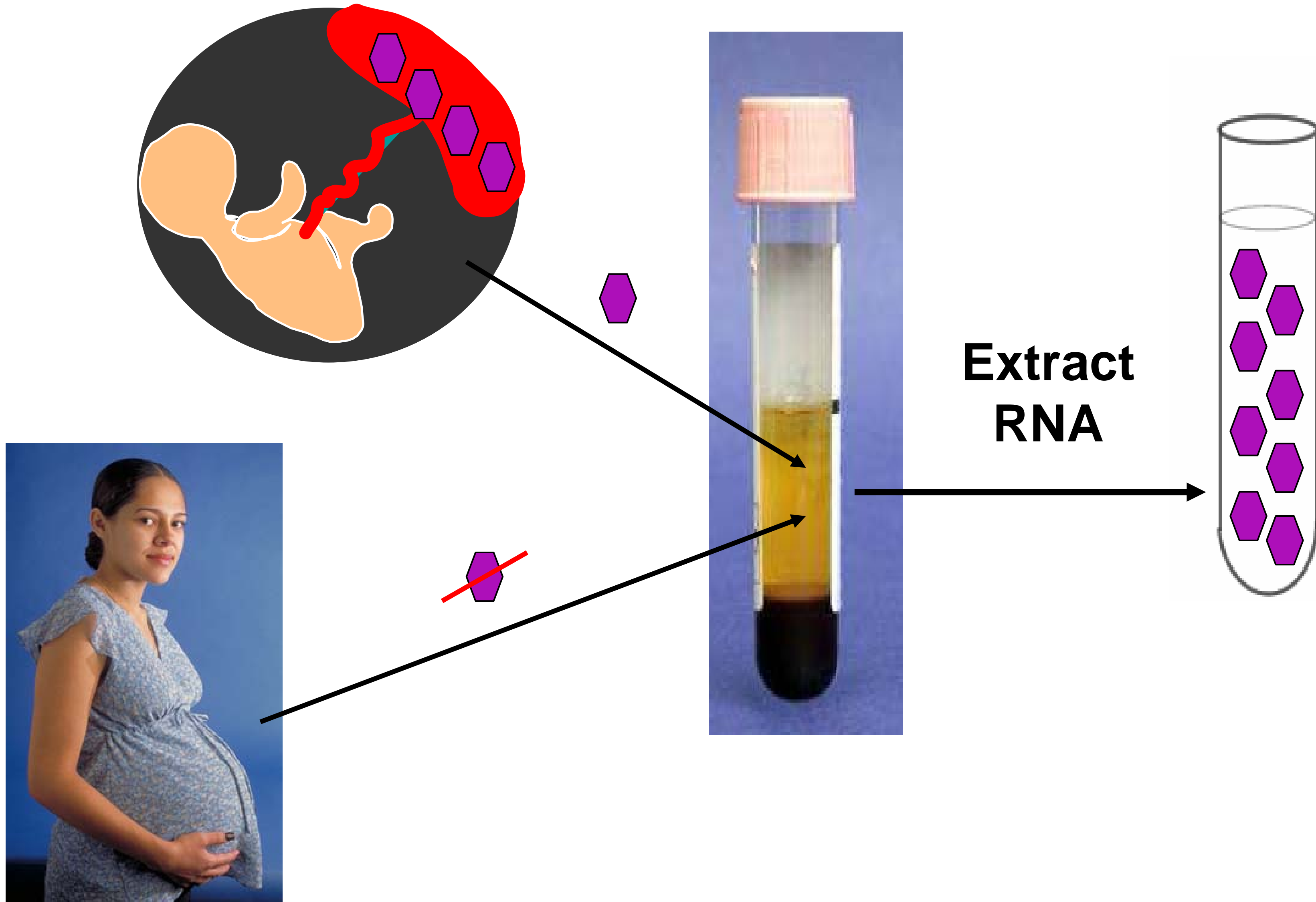
- **Recent major breakthroughs**

Quantitative analysis of SNPs in fetal specific mRNA transcripts
(Lo et al., PNAS 2007; Lo et al., Nature Medicine 2007; Maron et al, 2007)

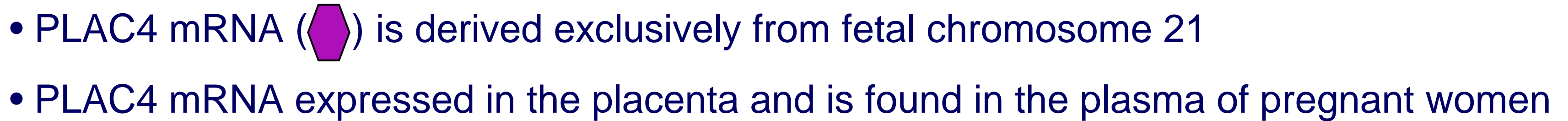
Epigenetic analysis (Tong et al., 2006; Old et al, 2007)

Proteomic analysis (e.g. Nagalla et al., 2007) Identification of novel protein biomarkers in maternal plasma associated with trisomy 21 pregnancies

Quantitative analysis of SNPs in fetal specific mRNA



Quantitative analysis of SNPs in fetal specific mRNA



- PLAC4 mRNA ( ) is derived exclusively from fetal chromosome 21
- PLAC4 mRNA expressed in the placenta and is found in the plasma of pregnant women

Normal fetus

Normal fetus

A G

Fetus with trisomy 21

A A G

Trisomy 21 fetus

1 : 1

Allele-A Allele-G

Intensity

Mass

A GT

Allele-A Allele-G

Intensity

Mass

A

A GT

2 : 1

- Correctly diagnosed fetal trisomy 21 in **90%** of +21 cases (n=10)
- Excluded diagnosis of trisomy 21 in **96.5%** of chromosomally normal controls (n=57)
- Fetus has to be informative for SNP analysed

Lo et al., Nature Med & PNAS, 2007

Advantages of testing cf fetal mRNA for DS testing and challenges for the future

- Transcripts are **fetal specific** and therefore independent of maternal contribution allowing copy numbers to be accurately quantified

- Diagnostic sensitivity and specificity from this study using one marker are comparable to multiple marker screening tests for DS

- Test appears to be insensitive to gestational age and can be offered early in pregnancy

- Multi centre large scale validation required

- Identification of more polymorphic loci to increase number of informative cases

- Expand testing to include other common fetal aneuploidies when fetal specific transcripts from chromosomes 18 and 13 are identified

Summary

DNA Extraction

• Enrichment of cf fetal DNA – will improve reliability of testing

• Any technique that can separate cf maternal and cf fetal DNA would revolutionise NIPD

Fetal sexing and single gene disorders

• Already in diagnostic use although development of more fetal specific markers required

• New techniques with increased levels of detection are being reported in research settings. Requires diagnostic validation

Aneuploidy

• Use of mRNA SNP allele ratio for testing for DS testing has been successfully applied in research setting. Requires diagnostic validation

• Epigenetic studies ongoing and proteomics studies are promising

Lack of quality control material and method standardisation