Technical challenges in non-invasive prenatal diagnosis

Cathy Meaney, Bhaneeta Lad, Gail Norbury.

NE Thames Regional Genetics Service

Great Ormond Street Hospital for Children
NHS Trust

Plan

- Background
- Current applications
- Challenges
- Experience
- Future

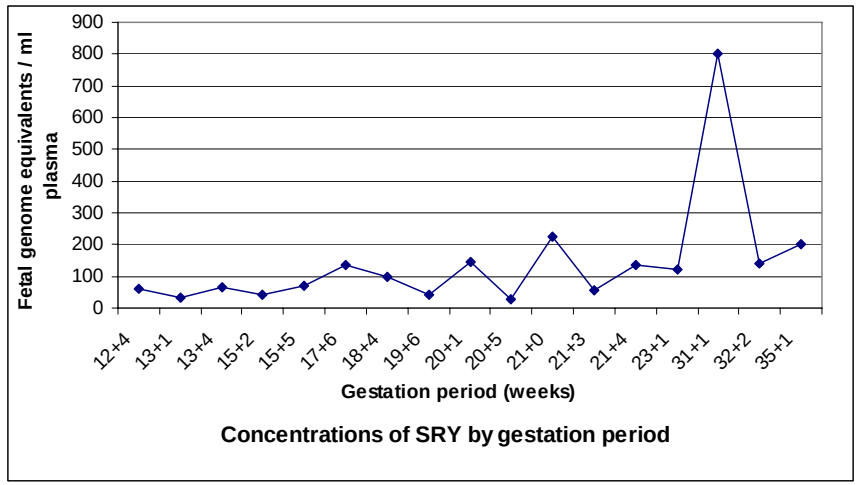
Non Invasive Prenatal Diagnosis

Goals of PND

Fetal Cells (1989)
Rare
Isolation
Longevity

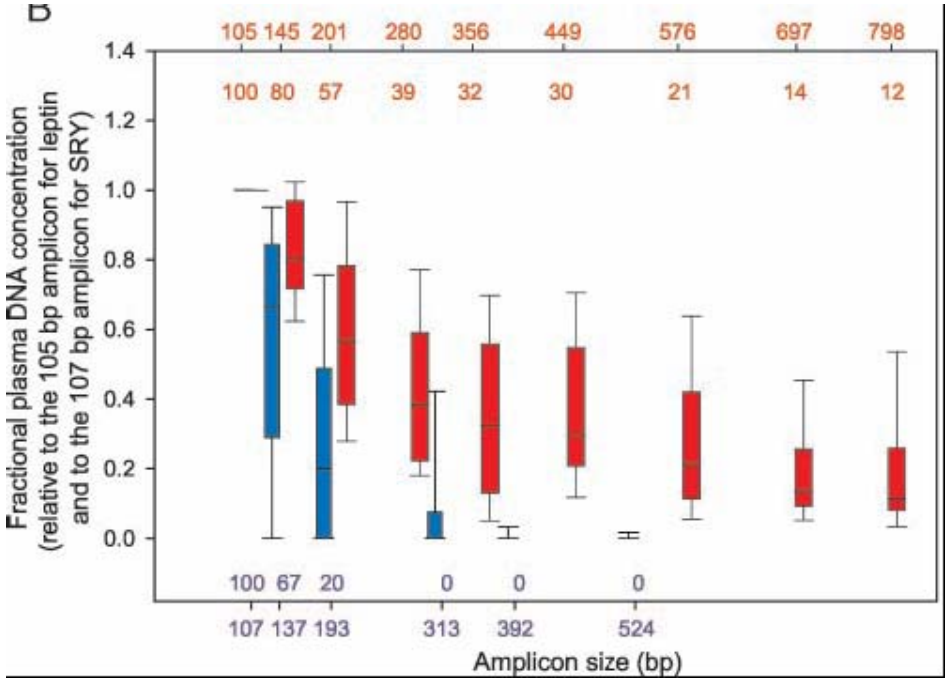
Cell Free DNA (1997)
Maternal haematopoietic cells
Fetal syncytiotrophoblasts

Gestation Period	Early	Late
Genome Equivalents (GE.) / ml maternal plasma	25.4	292.2
% fetal DNA of total DNA in maternal plasma	3.4%	6.2%

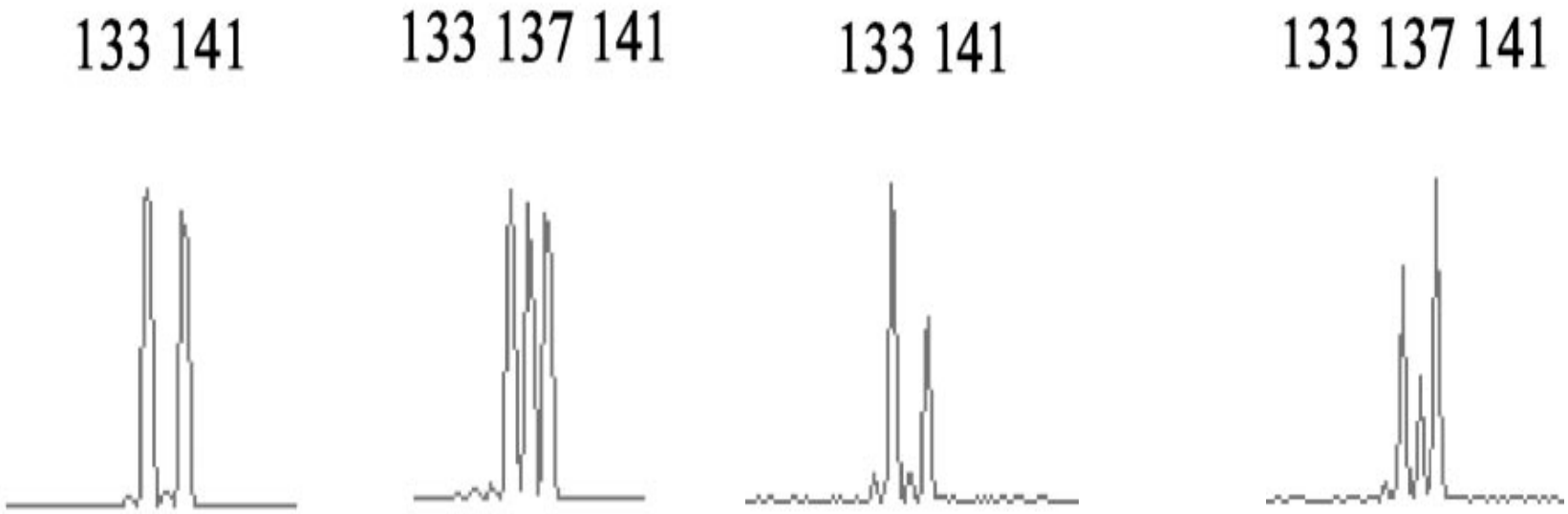


Cell free DNA

- Pregnancies with complications, EP
- Multiple pregnancies
- Clearance
- Mixture
  - Competition
  - Background
- Size



Chan et al, 2004



maternal genomic DNA	Fetal genomic DNA	Total circulatory DNA	Size fractioned circulatory DNA having a size of <300 bp i.e. detection of paternal 137 bp allele

Capillary electropherograms of D21S11 alleles (Li et al.2004)

Applications

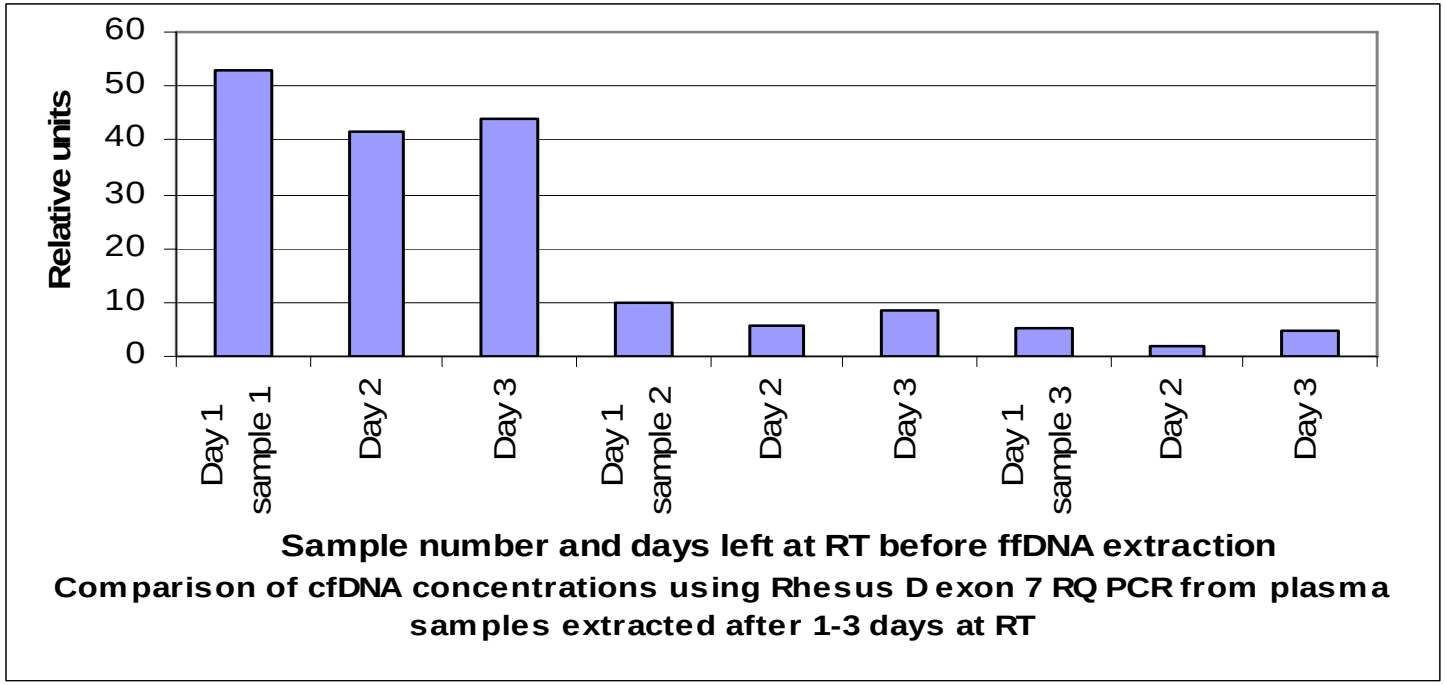
- **Blood typing**
  - Antenatal RhD-ve screening
- **Fetal sexing**
  - X-linked disease (DMD, ALD, XSCID, Hunter, OTC..)
  - CAH, AIS
- **De novo**
  - Ach
- **Paternal transmitted disorders**
  - DM (?size of material if expanded)
- **Recessive where high % compound heterozygotes**
  - CF, β-thalassaemia
- **(Aneuploidies)**

Challenges

- **Sensitivity**
  early detection when fetal DNA at low levels (2gE/PCR)
  suitable standards (plasmids, perfused placenta)
- **Specificity**
  Low amount of fetal DNA (1-12%) in high background of maternal DNA
- **Universal fetal-specific markers**
  to confirm presence of fetal DNA
- **Validation**
  Dynamic range (1-560 gE/ml fetal, 70->4000 gE/ml total)
- **Technology / Platforms**
  Real time PCR, SABER MALDI-TOF MS

Extraction parameters

- Separation (time, speed of separation, brake)
- +/-formaldehyde
- Serum or plasma
- Methods (Qiagen midi, mini, DRI-CST, MinElute)
- Volumes
- Automation – time, cost, yield
- Stability
- Storage

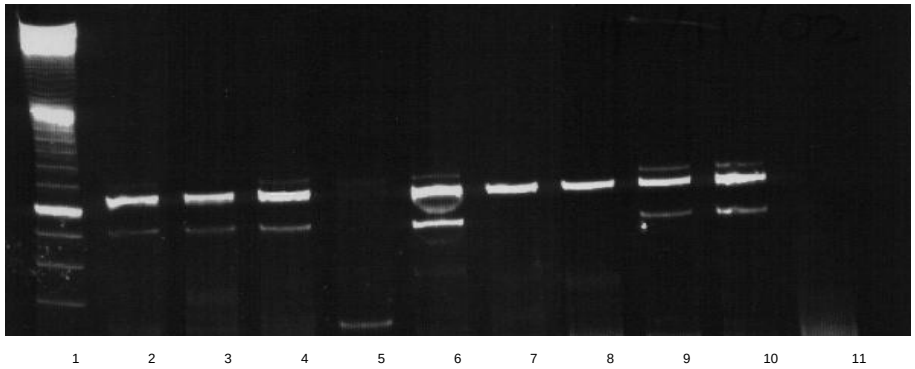


Comparison of cfDNA concentrations using Rhesus D exon 7 RQ PCR from plasma samples extracted after 1-3 days at RT

Sample storage

Extracts were successfully prepared up to 3 days after collection and following several months storage of plasma at –20^{0}C. However, it appears best to separate the plasma and analyse the extract within 24 hours.

Standard PCR



112 bp AMELB (Y)
106 bp AMELB (X)
93 bp SRY

Results

Of the total of 33 gels that were run, 10 (30.3%) were useable, 19 (57.6%) had male contamination, and 4 (12.1%) were too faint to interpret.

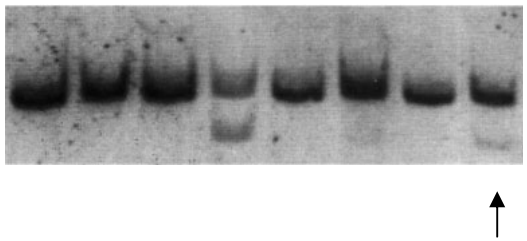
These 10 gels represented 80 runs for 56 samples (27 female and 29 male fetuses).

The useable results were then divided into those for the AMELB and SRY probes, to test the accuracy and efficiency of the test with these two probes.

	Sensitivity	**Specificity**	**PPV**
Amel B	29/38 **76.3%**	22/27 **81.5%**	29/34 **85.3%**
SRY	39/43 **90.7%**	19/30 **63.3%**	39/50 **78.0%**

Other attempts

- Cystic Fibrosis (p.Asn1303Lys) → ARCS
- Apert (p.Pro253Arg) → RED
- Thanatophoric dysplasia (p.Arg248Cys) → fluorescent primer & ARMs
- Literature
  - Nested PCR (SRY, DM1, AmelXY)
  - Touch down PCR. 9mls plasma, 34/40 cut out (Ach)



Ach. Hahn et al 2004

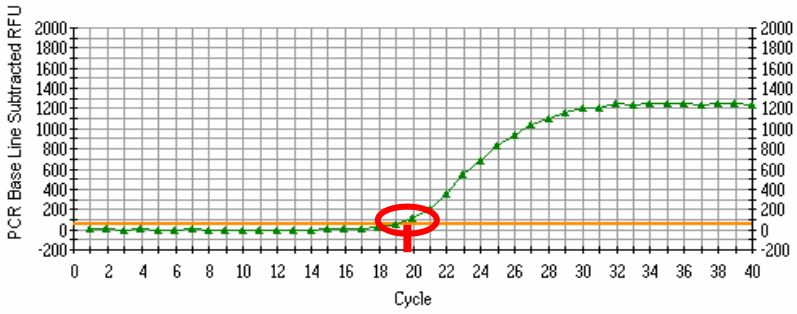
Real-Time PCR

- Closed system - contamination
- Probes – specificity, multiplex, sensitivity
- Melt curve analysis - products, efficiency
- Automation

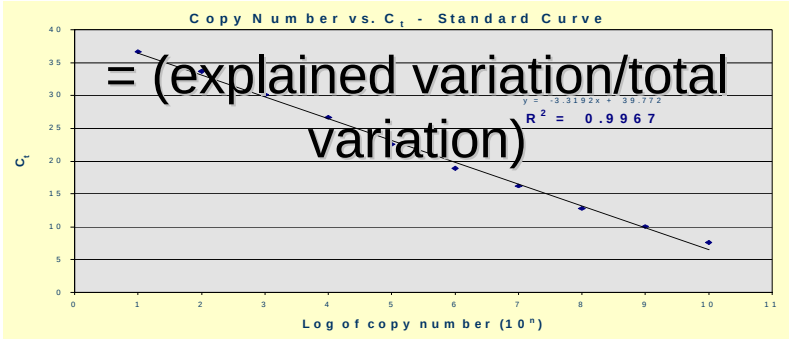
- Optimisation
- Cost reagents
- Multiplex

Threshold Cycle, C_T

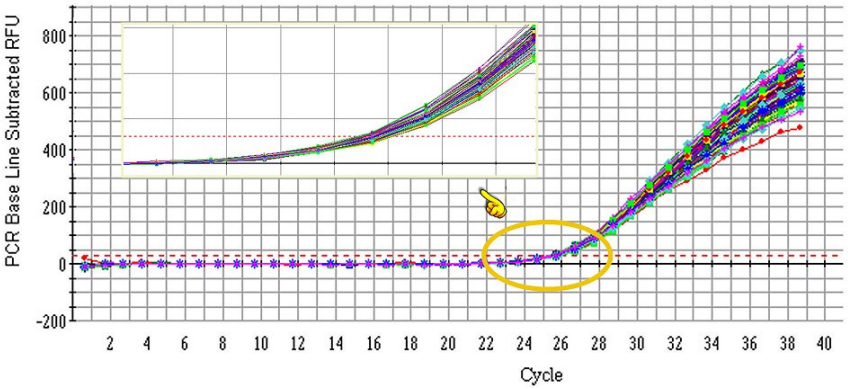
The point at which the fluorescence rises appreciably above background



r = is a measure of how well the actual data fit to the standard curve.



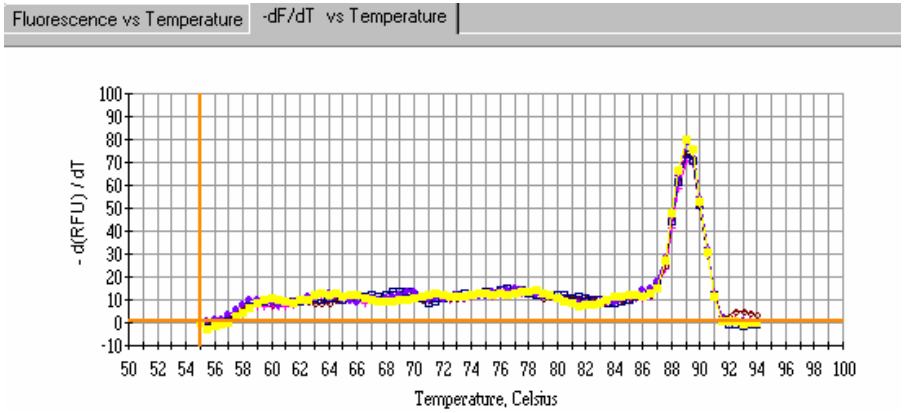
= (explained variation/total variation)



The slope of the standard curve can be directly correlated to the efficiency of the reactions:

Efficiency (η) = $[10^{(-1/\text{slope})}]$ - 1

Identify primer-dimers by melt curve to determine dynamic range



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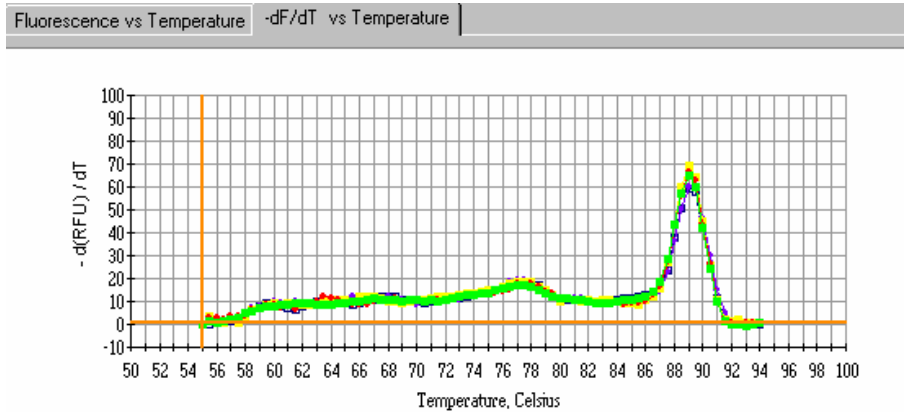

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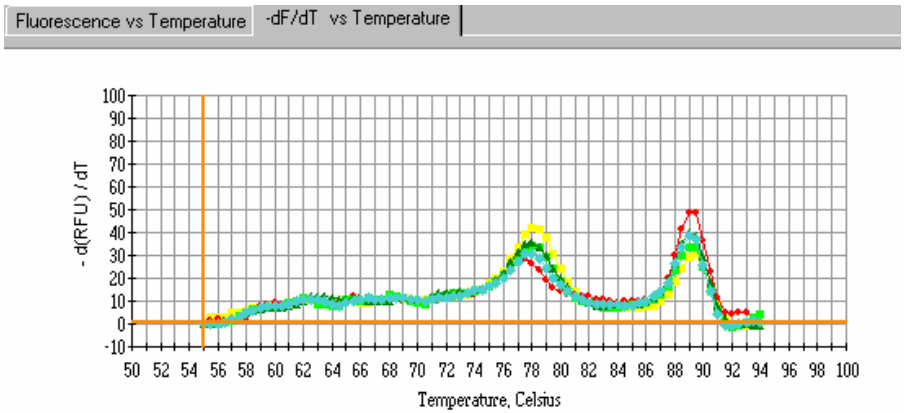

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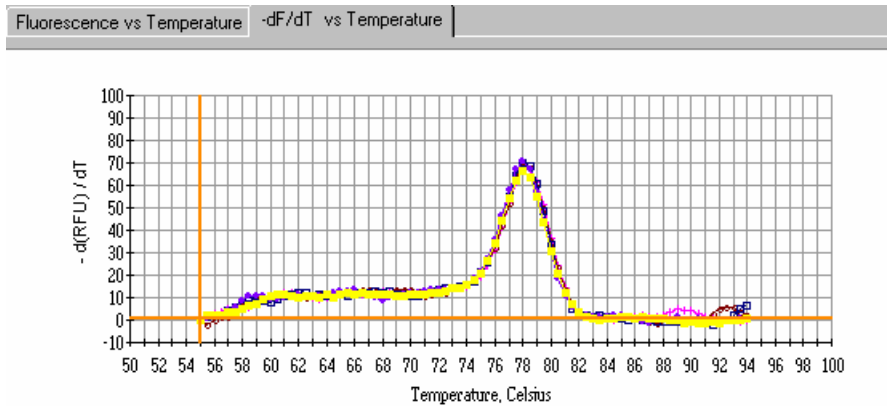


No template control

Multiplexing - Didn't work

Approach

Design assays for real-time PCR – primer and dual labelled probe
Check for secondary structure in product
Keep standard annealing temp
Set up individual assays and standard curves, 1st with Sybr Green then dual labelled probes using male DNA diluted in female to appropriate concentration range (0.01ng)
Combine as multiplex

Maximize Efficiency, Equalize Efficiency, Eliminate Cross-Reactivity

Problems

Increased cycles
Increase annealing time, try 3 step instead 2 step
Change Mg
Change reaction volume 25 to 50µl
Check multiplex with Sybr Green

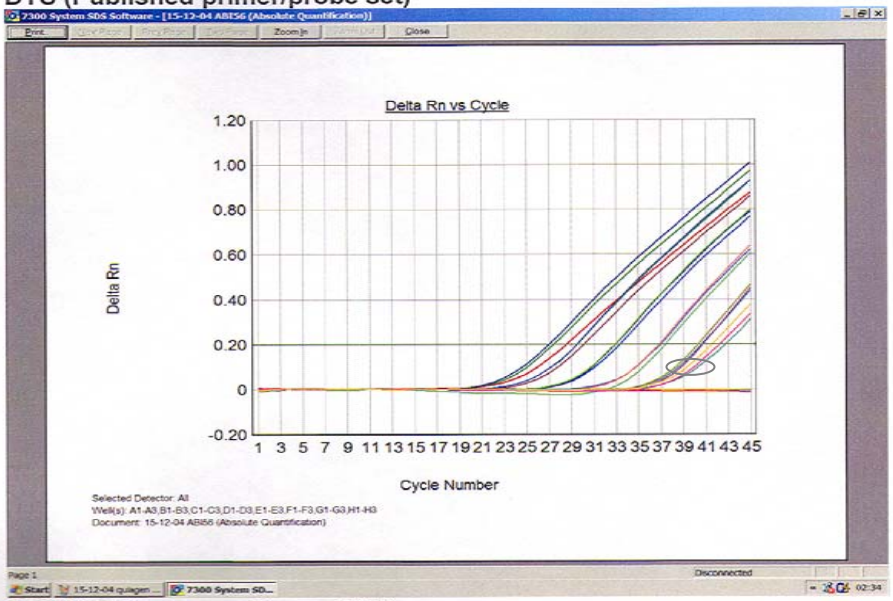
Interaction between primers & probes when multiplexed reduced sensitivity

Fetal sex determination using real time PCR-Taqman assays

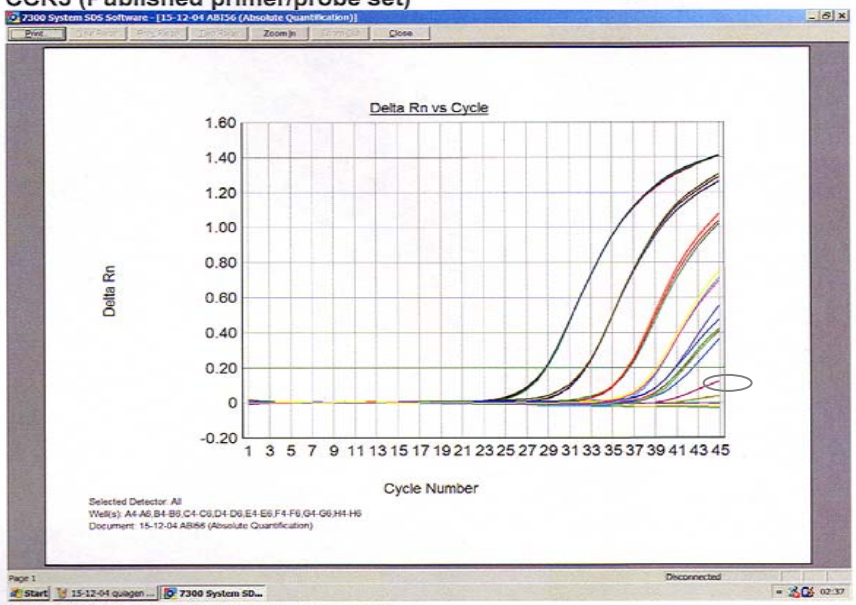
Platform	Y specific primers/probe	Control primers/probe	Source
iCycler	AMEL Y (FAM) & SRY (HEX)	AMEL X (ROX)	In-house design
iCycler	DYS14 (FAM)	CCR5 (FAM)	Published sequences
AB 7300	SRY (FAM)	CCR5 (FAM)	AB Assay-on-Demand Kits

AB 7300: Comparison of DYS/CCR5 assays & AB SRY/CCR5 Assay-on-Demand Taqman-MGB kits

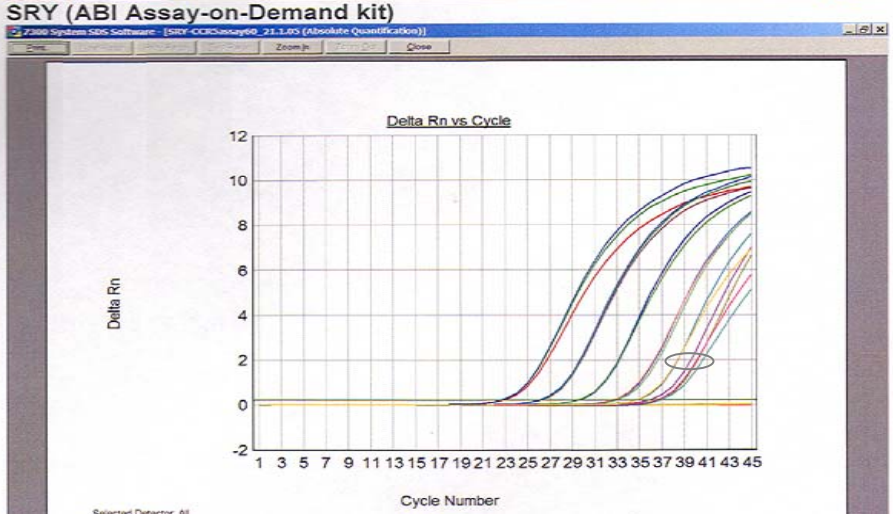
DYS (Published primer/probe set)



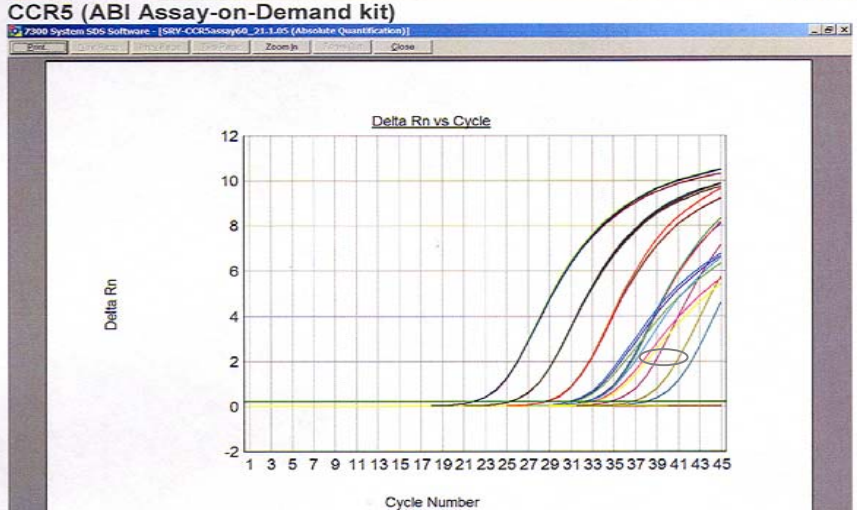
CCR5 (Published primer/probe set)



SRY (ABI Assay-on-Demand kit)



CCR5 (ABI Assay-on-Demand kit)



Result validation

- Positive result (PCR product with SRY)

  4/5 replicates with Ct < 40 cycles
- Negative result (no PCR amp with SRY)

  all replicates with Ct > 40 cycles
- Negative controls

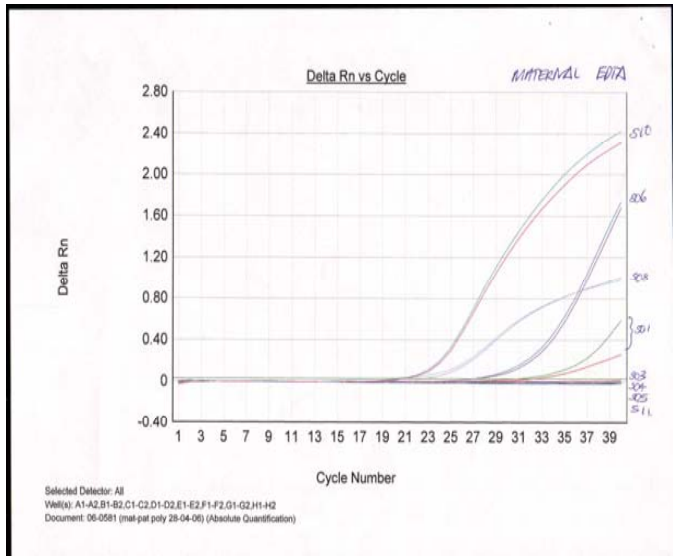
  no H20 control wells with Ct value < 40
- Positive controls

  all male replicates Ct < 40
- Slope of standard curves

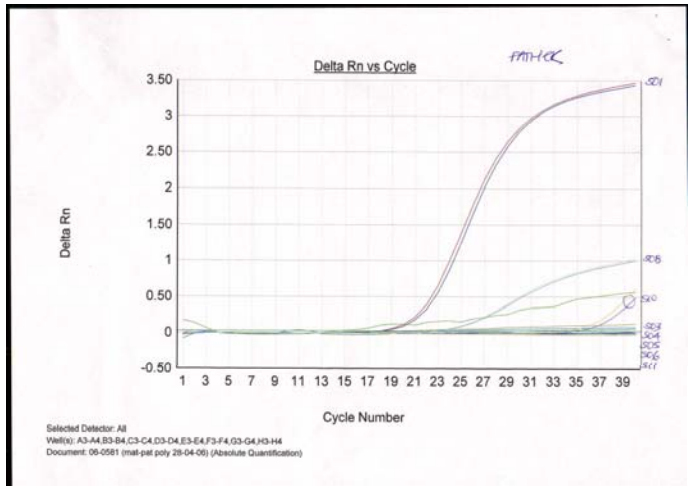
  between –3.3 and –4.5
- Repeat if discrepancies

Polymorphisms

- Test mother (& father) for 8 bi allelic makers
- Re-test mother and cfDNA for informative markers
- If no SRY in cfDNA but positive non-maternal marker assume evidence of ffDNA in cfDNA



Maternal marker informative (-ve) S03,4,5,11



Paternal marker informative (+ve) S01 (8, 10)

SAFE Workshop 2:

Showing the quality of performance of non-invasive prenatal genotyping in Europe

- 0.6 ml plasma (16/40) sent out at RT
- 18 partners received 5-6 samples
- Tested for RhD & SRY

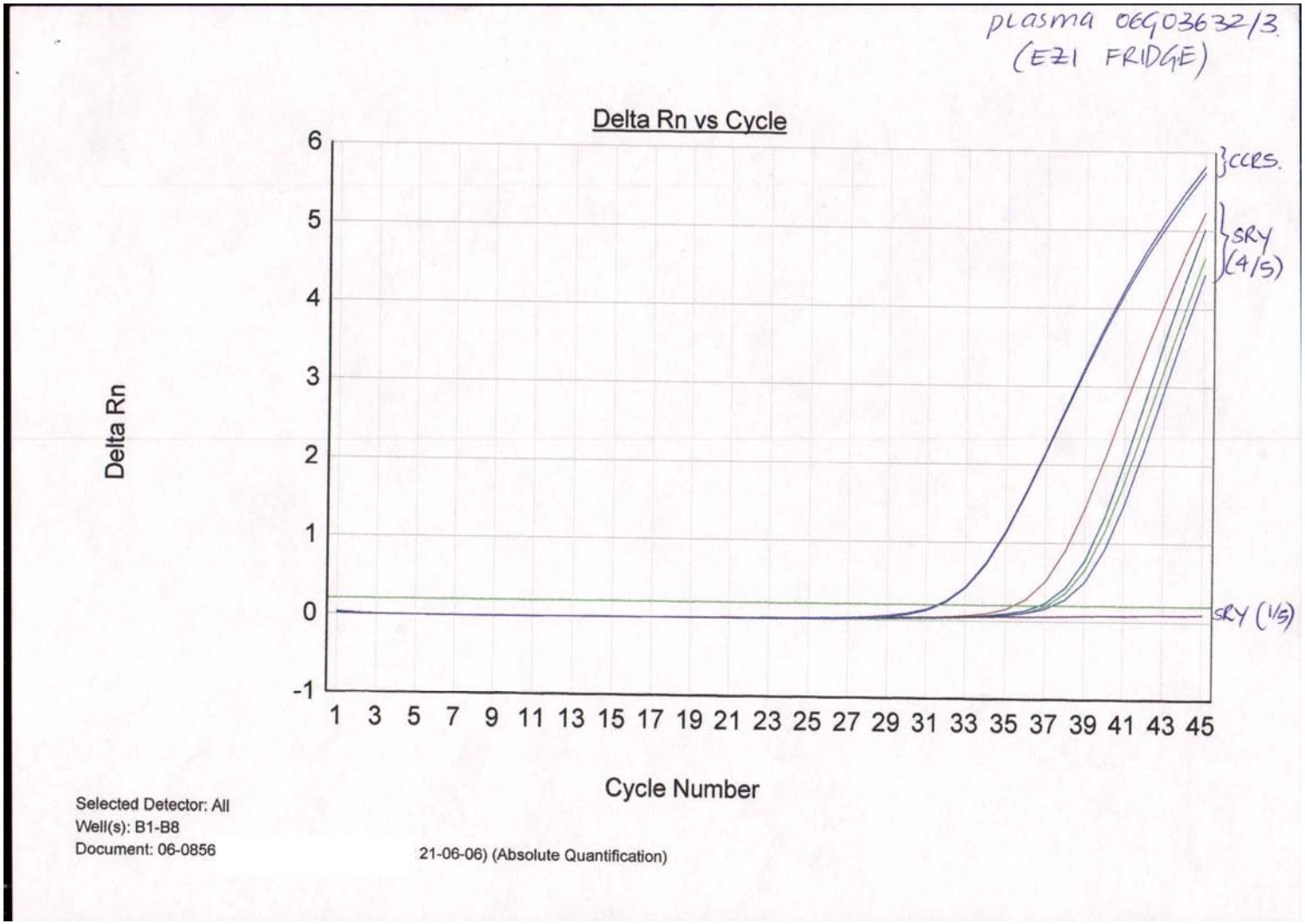
Results on 105 RhD and 109 SRY

- Correct results: 193 (90.2%)

- False results: 10 (4.7%)
  6 false positive and 4 false negative

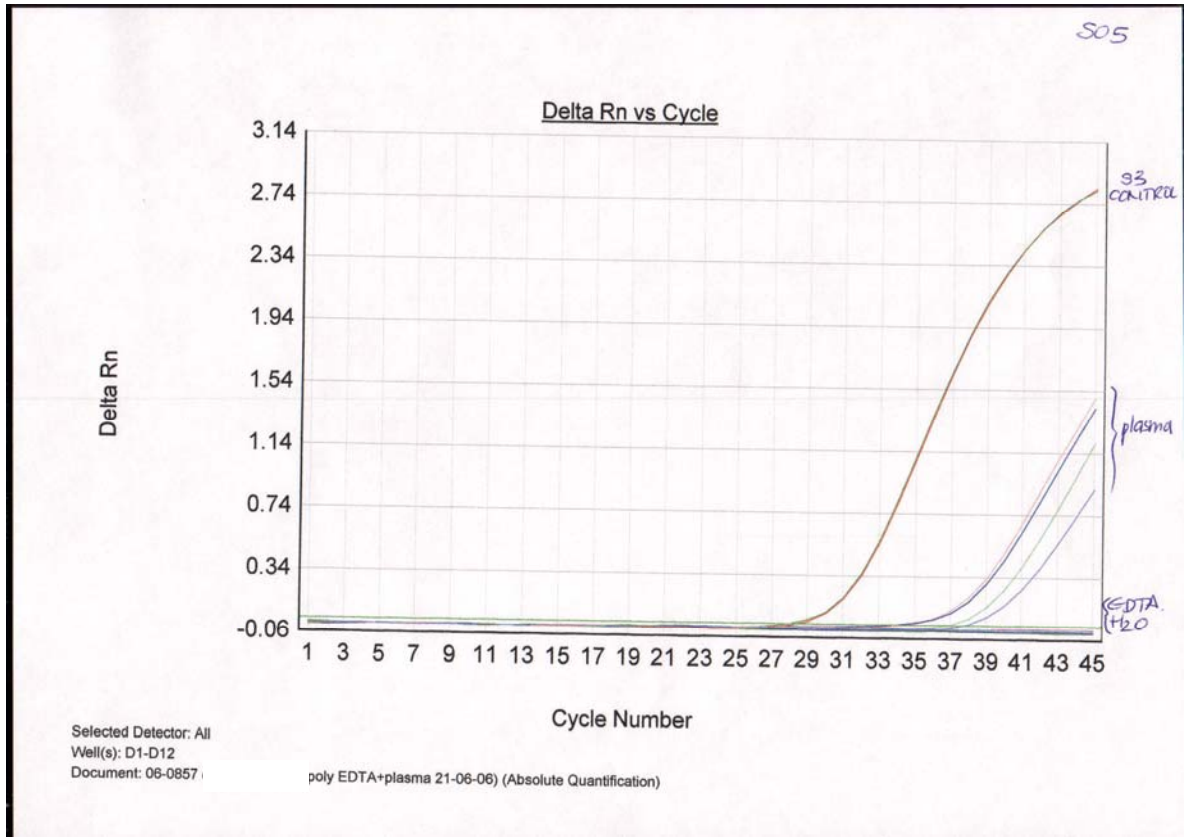
- Inconclusive results: 11 (5.1%)
  8 in positive samples and 3 in negative samples

Case 1. Male cfDNA – PND for ALD



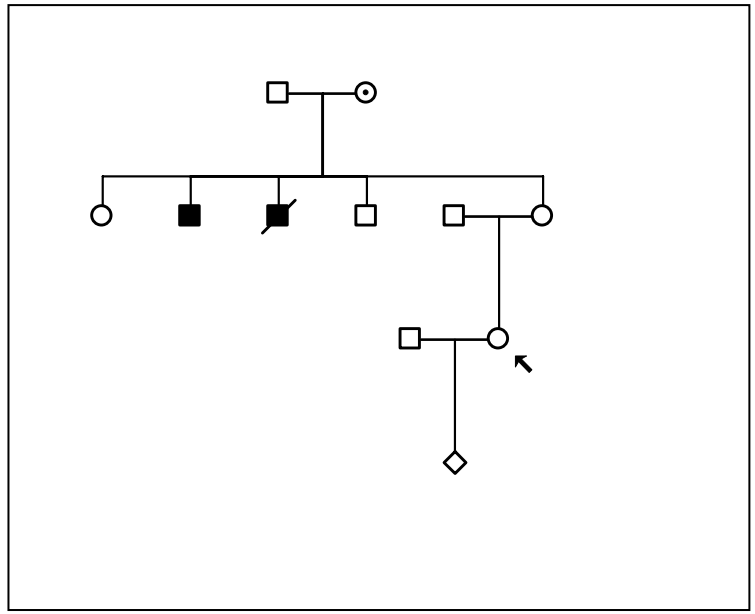
Case 2. Marker provides evidence of ffDNA

So1a	17q	23.6%
SO3	6q	16.4%
S04a	9	23.6%
S05b	20	27.3%
S06	1p	31%
S08b	1q	20.2%
S10a	18	25.5%
S11a	11	25.5%



Case 3. Duchenne muscular dystrophy

- Mutation not known
- Consultand would like PND
- Affecteds deceased
- Gm won’t give sample
- Options



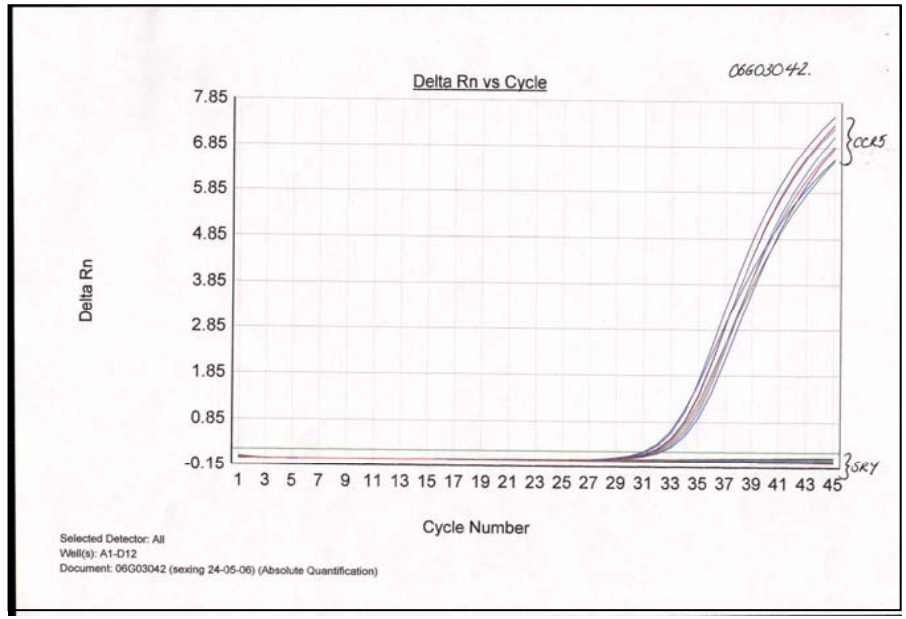
- 13/40 cfDNA – SRY not detected
- 4 markers not informative
- Repeat at 14/40 – SRY not detected
- U/Scan shows female
- On-going

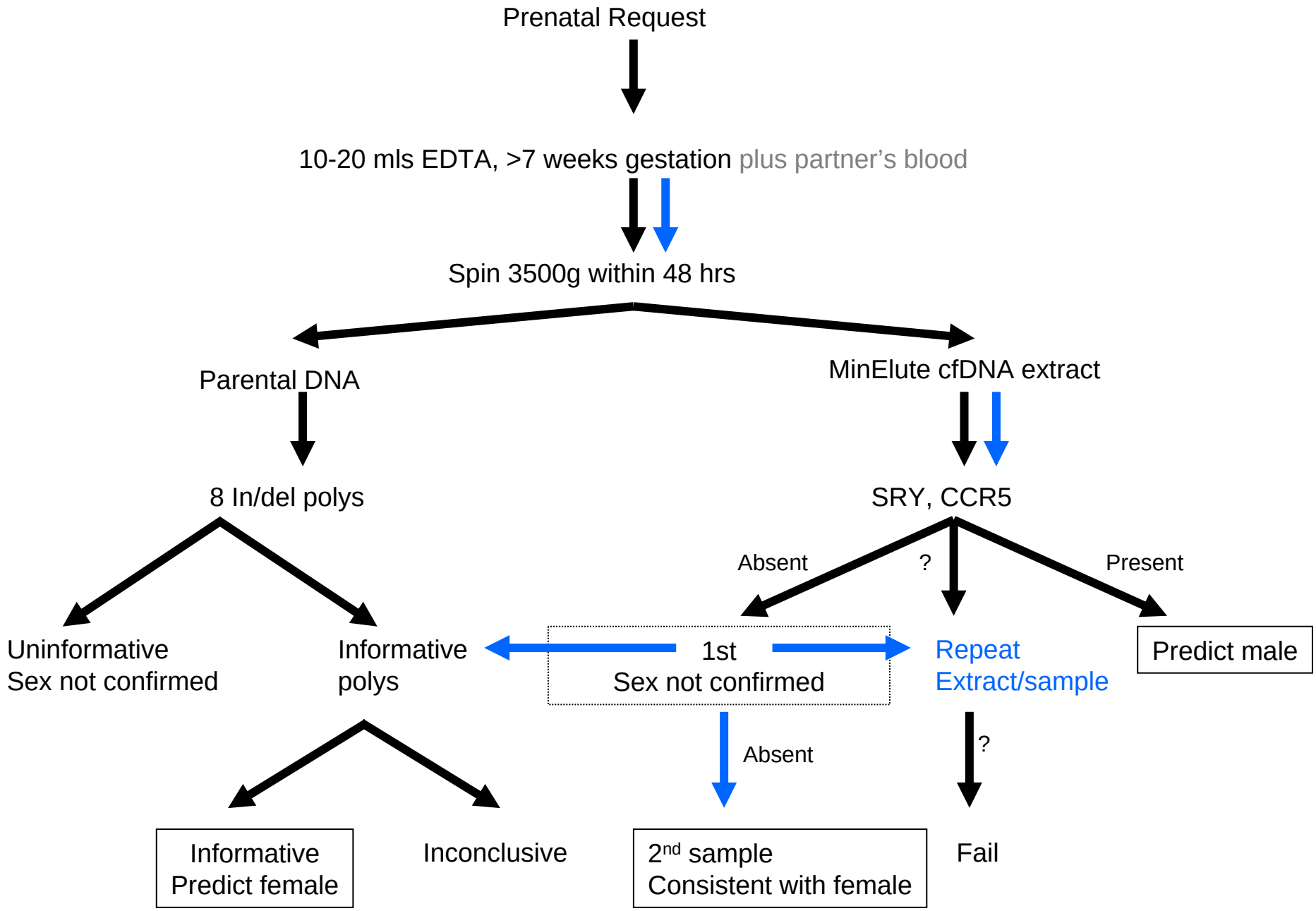
SRY	FAM	TAMRA	0.200000	3	15					
CCR5	FAM	TAMRA	0.200000	3	15					

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered	Tm
A1	06G03042	SRY	Unknown	Undet.						
A2	06G03042	SRY	Unknown	Undet.						
A3	06G03042	SRY	Unknown	Undet.						
A4	06G03042	SRY	Unknown	Undet.						
A5	06G03042	SRY	Unknown	Undet.						
A7	06G03042	CCR5	Unknown	31.58	0.129	180.62	171.18	13.344		
A8	06G03042	CCR5	Unknown	31.77	0.129	161.74	171.18	13.344		
B1	06G03042/2	SRY	Unknown	Undet.						
B2	06G03042/2	SRY	Unknown	Undet.						
B3	06G03042/2	SRY	Unknown	Undet.						
B4	06G03042/2	SRY	Unknown	Undet.						
B5	06G03042/2	SRY	Unknown	Undet.						
B7	06G03042/2	CCR5	Unknown	31.01	0.095	255.75	245.82	14.051		
B8	06G03042/2	CCR5	Unknown	31.14	0.095	235.88	245.82	14.051		
C1	06G03042/3	SRY	Unknown	Undet.						
C2	06G03042/3	SRY	Unknown	Undet.						
C3	06G03042/3	SRY	Unknown	Undet.						
C4	06G03042/3	SRY	Unknown	Undet.						
C5	06G03042/3	SRY	Unknown	Undet.						
C7	06G03042/3	CCR5	Unknown	32.10	0.480	132.16	165.75	47.512		
C8	06G03042/3	CCR5	Unknown	31.42	0.480	199.35	165.75	47.512		
D1	06G03042/4	SRY	Unknown	Undet.						
D2	06G03042/4	SRY	Unknown	Undet.						
D3	06G03042/4	SRY	Unknown	Undet.						
D4	06G03042/4	SRY	Unknown	Undet.						
D5	06G03042/4	SRY	Unknown	Undet.						
D7	06G03042/4	CCR5	Unknown	32.26	0.259	119.87	134.72	21.005		
D8	06G03042/4	CCR5	Unknown	31.90	0.259	149.58	134.72	21.005		
E1	SRY H20 BLANK	SRY	NTC	Undet.						
E2	SRY H20 BLANK	SRY	NTC	Undet.						
E3	SRY H20 BLANK	SRY	NTC	Undet.						
E7	CCR5 H2O BL...	CCR5	NTC	Undet.						
E8	CCR5 H2O BL...	CCR5	NTC	Undet.						
E9	CCR5 H2O BL...	CCR5	NTC	Undet.						

06G03042 (sexing 24-05-06)

Page 2 of 6 (05/24/06 14:01:04)




Prenatal Request
10-20 mls EDTA, >7 weeks gestation plus partner's blood
Spin 3500g within 48 hrs
Parental DNA
MinElute cfDNA extract
8 In/del polys
SRY, CCR5
Absent
?
Present
Uninformative
Sex not confirmed
Informative
polys
1st
Sex not confirmed
Repeat
Extract/sample
Predict male
Absent
?
Informative
Predict female
Inconclusive
2nd sample
Consistent with female
Fail

Summary of challenges

- Robustness (multiplex)
- Fetal Markers
- Future
  - Enrichment of <350 bp cfDNA (microfluidics)
  - Quantitation (management of delivery)
  - Panel of disease-specific markers (cardiac, renal, skeletal)

Acknowledgements

Clinical

- Lyn Chitty, FMU,UCH

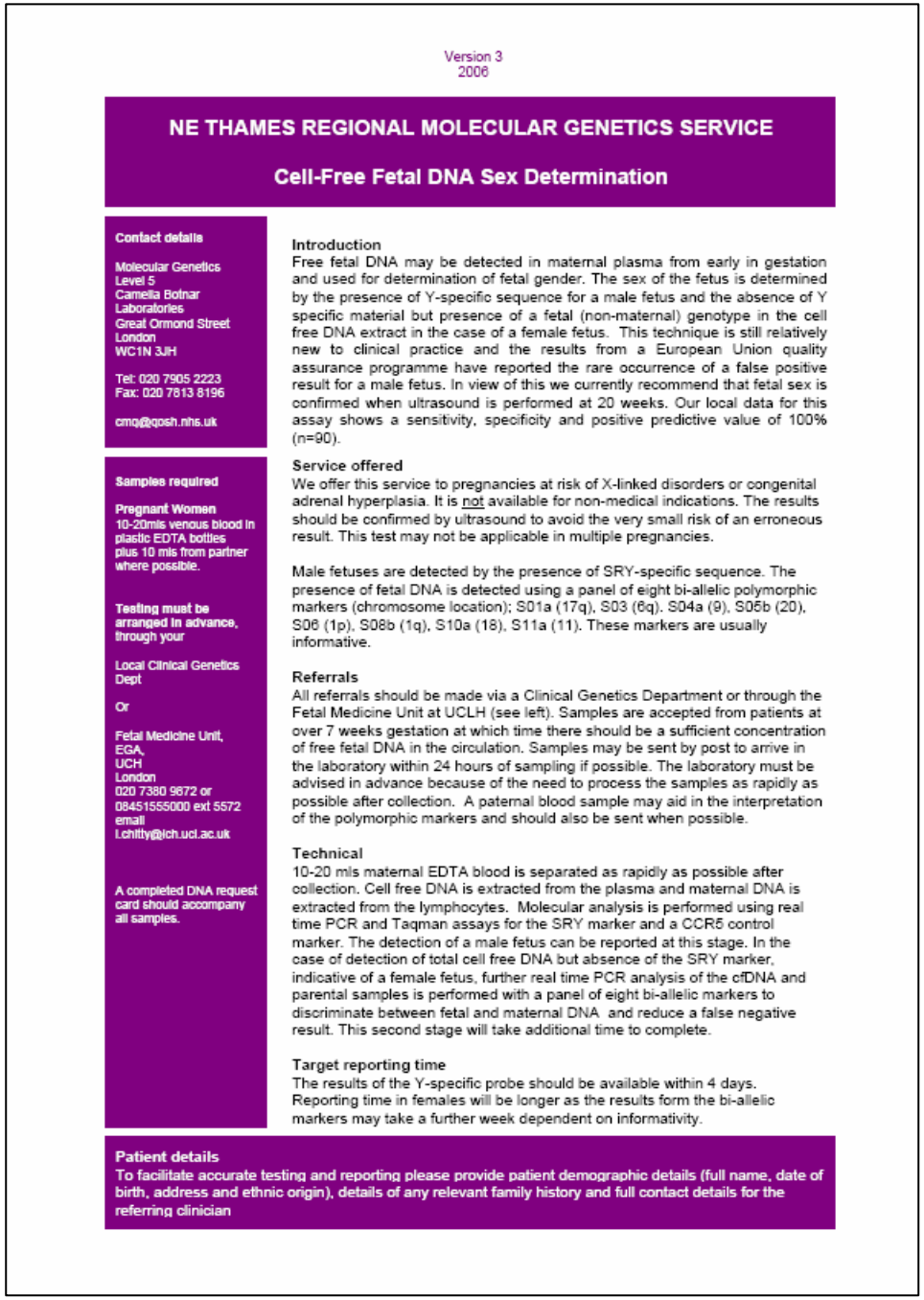
Technical

- Kirsten Finning, IBGRL

Funding

- ICH Pump prime grant
- EU SAFE

http://www.gosh.nhs.uk/clinserv/molecular_genetics.htm



Version 3
2006

NE THAMES REGIONAL MOLECULAR GENETICS SERVICE

Cell-Free Fetal DNA Sex Determination

Contact details

Molecular Genetics
Level 5
Camelia Botnar
Laboratories
Great Ormond Street
London
WC1N 3JH

Tel: 020 7905 2223
Fax: 020 7813 8196

cmq@gosh.nhs.uk

Samples required

Pregnant Women
10-20mls venous blood in plastic EDTA bottles plus 10 mls from partner where possible.

Testing must be arranged in advance, through your

Local Clinical Genetics Dept

Or

Fetal Medicine Unit, EGA, UCH
London
020 7380 9872 or 08451555000 ext 5572
email l.chitty@ich.ucl.ac.uk

A completed DNA request card should accompany all samples.

Introduction
Free fetal DNA may be detected in maternal plasma from early in gestation and used for determination of fetal gender. The sex of the fetus is determined by the presence of Y-specific sequence for a male fetus and the absence of Y specific material but presence of a fetal (non-maternal) genotype in the cell free DNA extract in the case of a female fetus. This technique is still relatively new to clinical practice and the results from a European Union quality assurance programme have reported the rare occurrence of a false positive result for a male fetus. In view of this we currently recommend that fetal sex is confirmed when ultrasound is performed at 20 weeks. Our local data for this assay shows a sensitivity, specificity and positive predictive value of 100% (n=90).

Service offered
We offer this service to pregnancies at risk of X-linked disorders or congenital adrenal hyperplasia. It is not available for non-medical indications. The results should be confirmed by ultrasound to avoid the very small risk of an erroneous result. This test may not be applicable in multiple pregnancies.

Male fetuses are detected by the presence of SRY-specific sequence. The presence of fetal DNA is detected using a panel of eight bi-allelic polymorphic markers (chromosome location): S01a (17q), S03 (6q). S04a (9), S05b (20), S06 (1p), S08b (1q), S10a (18), S11a (11). These markers are usually informative.

Referrals
All referrals should be made via a Clinical Genetics Department or through the Fetal Medicine Unit at UCLH (see left). Samples are accepted from patients at over 7 weeks gestation at which time there should be a sufficient concentration of free fetal DNA in the circulation. Samples may be sent by post to arrive in the laboratory within 24 hours of sampling if possible. The laboratory must be advised in advance because of the need to process the samples as rapidly as possible after collection. A paternal blood sample may aid in the interpretation of the polymorphic markers and should also be sent when possible.

Technical
10-20 mls maternal EDTA blood is separated as rapidly as possible after collection. Cell free DNA is extracted from the plasma and maternal DNA is extracted from the lymphocytes. Molecular analysis is performed using real time PCR and Taqman assays for the SRY marker and a CCR5 control marker. The detection of a male fetus can be reported at this stage. In the case of detection of total cell free DNA but absence of the SRY marker, indicative of a female fetus, further real time PCR analysis of the cfDNA and parental samples is performed with a panel of eight bi-allelic markers to discriminate between fetal and maternal DNA and reduce a false negative result. This second stage will take additional time to complete.

Target reporting time
The results of the Y-specific probe should be available within 4 days. Reporting time in females will be longer as the results form the bi-allelic markers may take a further week dependent on informativity.

Patient details
To facilitate accurate testing and reporting please provide patient demographic details (full name, date of birth, address and ethnic origin), details of any relevant family history and full contact details for the referring clinician