Non-invasive determination of fetal blood groups from maternal Plasma

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Certified according to DIN ISO 9001:2000

Germany-Austria-China Academic Exchange Conference (Shenzhen) on Blood Group
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Hemolytic disease of the fetus/ newborn

- anemia
- hyperbilirubinemia
- hydrops fetalis
- enlargement of liver and spleen

cause:

hemolytic anemia due to maternal IgG alloantibodies
# Antibody specificities

<table>
<thead>
<tr>
<th>Antibody Specificities</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO-antibodies</td>
<td>Anti-M</td>
</tr>
<tr>
<td>Anti-D</td>
<td>Anti-N</td>
</tr>
<tr>
<td>Anti-c</td>
<td>Anti-s</td>
</tr>
<tr>
<td>Anti-Kell</td>
<td>Anti-U</td>
</tr>
<tr>
<td>Anti-E</td>
<td>Anti-PP(_1)p(_k)</td>
</tr>
<tr>
<td>Anti-Jk</td>
<td>Anti-Di(b)</td>
</tr>
<tr>
<td>Anti-Js(a)</td>
<td>Anti-LAN</td>
</tr>
<tr>
<td>Anti-Ku</td>
<td>Auto-antibodies (rare)</td>
</tr>
<tr>
<td>Anti-Fy(a)</td>
<td></td>
</tr>
</tbody>
</table>
HDN: Diagnosis and follow up

- Antibody-titer during pregnancy
- ultrasound
- Doppler sonographie of the arteria cerebri media
Aszites
Hb 3,8 g/dl

Aszites
Hb 1,2 g/dl

Hydrops placentae
Hb 2,1 g/dl

Hepato-Splenomegalie
Hb 4,2 g/dl
NONINVASIVE DIAGNOSIS OF FETAL ANEMIA DUE TO MATERNAL RED-CELL ALLOIMMUNIZATION

OF THE BLOOD VELOCITY IN ANEMIC FETUSES
Rh-Prophylaxis

Immunization against RhD can be prevented if
- before immunization
- an appropriate amount of
- anti-D immunoglobulin
is given.
ABO, RhD Ab-Screen

Ab-Screen

12

24

28

32

40

72 h

28-30 SSW

300 µg

Anti-D IgG

05.06.08

Anti-D IgG

300 µg

Anti-D IgG
Figure 12.8  Registered deaths due to haemolytic disease of the fetus and newborn in England and Wales, 1977–90 (data for 1977–89, see Clarke and Mollison (1989); for 1989, see Hussey and Clarke (1991); for 1990, C.A. Clarke, personal communication.) ●, Total deaths due to anti-D; ○, deaths due to anti-D in which the mother had been treated with anti-D immunoglobulin following previous deliveries; x, deaths due to antibodies other than anti-D (mainly anti-c and anti-K); for details see text.
**Frequency of Anti-D alloimmunization**

Bowman J: Transfusion 2003;43:1661-1666

- Pregnant women without prophylaxis: 13.2% per D+ pregnancy
- Pregnant women with postdelivery Rh prophylaxis: 1.8% per D+ pregnancy
- Pregnant women with antenatal and postnatal Rh prophylaxis: 0.14% per D+ pregnancy
Why non-invasive determination of the fetal D-Status?

Anti-D immunoglobulin:
• statistically not indicated in 38.5% of all D-negative pregnancies in Whites (17.3% in Germany)
• human material
• potentially infectious
• short supply
• costs
Some RhD Details

- D-positive
- Weak D-antigen density (D-alleles: weak D, partial D)
- D-negative

Frequent molecular types
- Weak D type 1-3
- Partial D-category III-VII
- Partial D: DAU
- DEL (K409K)
- $RHD_\psi$, $RHD-CD-D$ (Serology D-)
Rearranged RH haplotypes

Current prenatal diagnosis requires invasive procedures

Aneuploidy
Single gene disorders
Haemoglobinopathies

1% risk of miscarriage
Not possible before 11 weeks’
Other sources of fetal tissue for PND

**Fetal cells in maternal circulation**
- erythroblasts
- trophoblastic cells
- leucocytes

Difficult to isolate, certain cells may persist post pregnancy

**Cell free fetal DNA in the maternal circulation**
- Detectable from 5 weeks’
- Cleared from circulation within 30 minutes of delivery
- 3 – 6% of total circulating cell free DNA
Fetal *RHD* genotyping milestones

1993  Lo YM et al. Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers (cells).

1998  Faas BH et al. and Lo YM et al.: Detection of fetal *RHD*-specific sequences in maternal plasma.

2002  Finning KM et al. and Legler TJ et al.: Improved fetal *RHD* genotyping to distinguish *RHD* from *RHDψ*.

2004  Soussan AA et al. First large scale fetal *RHD* screening study using automation presented.

2007  Legler TJ and SAFE partners: Workshop report on the extraction of fetal DNA from maternal plasma published in *Prenat Diag* 27(9): 824-9
Cell-free fetal (cff) DNA in maternal plasma

Where does it come from?

- Apoptosis of fetal cells?
- Plazenta! No fix barrier

Trophoblast

(Guibert et al., Hum. Repr., 2003; 18 (8), 1733-36)
Rapid clearance of fetal DNA from maternal Plasma

T1/2 = 16 minutes (range 4-30)
Sample preparation and storage

If possible, separate plasma from cells within 48 (24) hours and freeze plasma <-70°C

use EDTA rather than serum

exclude hemolytic samples
Genetic analysis without risk

Molecular genetic prenatal diagnosis requires currently amniocentesis which is associated with a risk for the fetus. The aim of the co-operation between Sequenom and Qiagen is, to develop a technology for the enrichment of free fetal DNA from maternal plasma in order to support Sequenom’s development of standardized non-invasive Methods for non-invasive prenatal genetic diagnosis.
The Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network

Chair of steering committee
Neil Avent (neil.avent@uwe.ac.uk)
Scientific Director
Sinuhe Hahn (shahn@uhbs.ch)

Network of excellence sponsored under the EU’s Framework programme 6
‘Life Sciences, Genomics and Biotechnology for Health’
Aim

To implement routine, cost effective non-invasive prenatal diagnosis (NIPD) and neonatal screening through the creation of long-term partnerships within & beyond the European community.

March 2004 – February 2009
50 partners from 19 countries
12 million euros
If you would like more information about this project, or would like to help by collecting samples, please email us at

safeproject@warwick.ac.uk

Alternatively please visit our website

www.safenoe.org

05.06.08
Challenges: Invasive testing vs. NI PD

Invasive test
- Large amount of celluar DNA
- Pure cell population from the fetus
- Quantity of DNA usually not relevant for molecular genetic analysis

NIPD from cff DNA in maternal plasma
- Mixture of fetal and maternal DNA
- Fluctuation of fetal DNA
- Increase of fetal DNA during pregnancy (timepoint)
Concentration of free fetal DNA

1. Trimester: 23,1 (0-265) geq/ml (n=221)
2. Trimester: 32,4 (0-346) geq/ml (n=677)
3. Trimester: 77,7 (0-391) geq/ml (n=121)

geq=genome equivalents
The free fetal DNA concentration fluctuates

Total DNA in non-pregnant women: 1,9 – 67,9
(n=13, 3-6 different days, mean 13,5)

Total DNA in pregnant women: 1,3 - 130,5
(n=16, 3 different days, mean: 21,5)

cff DNA in maternal plasma: 1,4 - 4,5
(n=10, 3 different days, mean: 2,2)
I VDD 98/79/EC ANNEX I: ESSENTIAL REQUIREMENTS (3)

Performances stated by the manufacturer

- analytical sensitivity (lower limit of detection)
- diagnostic sensitivity
- analytical specificity
- diagnostic specificity
- accuracy
- repeatability, intra- and inter-assay variation
- reproducibility, including control of known relevant interference, and limits of detection
IVDD 98/79/EC ANNEX I:
ESSENTIAL REQUIREMENTS (3)

The traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order.

batch release criteria have to be defined
3.2. Additional requirements for nucleic acid amplification techniques (NAT)

3.2.1. For target sequence amplification assays, a functionality control for each test sample (internal control) shall reflect the state of the art. This control shall as far as possible be used throughout the whole process, i.e. extraction, amplification/hybridisation, detection.
SAFE: *RHD NIPD Standardisation workflow*

1. A reference measurement procedure was determined through a wet workshop.
2. The reference protocol was adapted in collaborative multi-center experiments and discussion workshops.
3. A reference material of a higher order for the determination of the analytical sensitivity was agreed in a workshop.
4. Diagnostic sensitivity and specificity were determined for the most promising technology platforms in large-scale single-centre studies.
5. A Quality Assurance scheme for gauging levels of repeatability, consistency and uniformity was established.
6. Agreement was obtained in a discussion workshop that the internal control is not mandatory for RHD NIPD screening.
DNA-Extraction Methods: Principles

Binding to solid-phase
- anion-exchange
- silica-based
- specific probes

Solid phase
- membrane
- particles
## Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magna Pure</td>
<td>2</td>
</tr>
<tr>
<td>Tecan Genesis 150/4/in-house</td>
<td>1</td>
</tr>
<tr>
<td>MDx</td>
<td>1</td>
</tr>
<tr>
<td>M48</td>
<td>1</td>
</tr>
<tr>
<td>EZ1</td>
<td>1</td>
</tr>
<tr>
<td>QIAamp DNA Mini Kit</td>
<td>7</td>
</tr>
<tr>
<td>QIAamp DNA Midi Kit</td>
<td>1</td>
</tr>
<tr>
<td>QIAamp DSP Virus Kit</td>
<td>1</td>
</tr>
<tr>
<td>High Pure Roche</td>
<td>1</td>
</tr>
<tr>
<td>CST Genome DNA</td>
<td>1</td>
</tr>
<tr>
<td>Hybridisation capture in-house</td>
<td>1</td>
</tr>
</tbody>
</table>
Reference measurement procedure

QIAamp DSP Virus Kit (Qiagen, Hilden, Germany), Cat. No. 60704

http://www.safenoe.org/protocols

Mean pg/ml

Pool
- Red: 1
- Green: 2
- Blue: 3

HP: High Pure PCR Template Preparation Kit, MINI: QIAamp DNA Blood Mini Kit, CST: CST genomic DNA purification Kit, MB: in-house magnetic bead separation method, MIDI: QIAamp DNA Blood Midi Kit

Automated DNA extraction methods

MP: Magnapure Roche, Tecan: Tip-Extraction, MDx, M48, EZ1 instruments from QIAGEN

Automated methods, pool 3: DNA amount for PCR

RHD NI PD Technology platforms assessed (larger scale)

QIAamp Blood Kit (Qiagen, Hilden, Germany) n=851

MagnaPure (Roche Diagnostics, Basel, Switzerland) n=1257
Van der Schoot CE et al. Transfus Clin Biol 2006;13:53-7

Ampliprep (Roche Diagnostics) n=545
Minon JM et al. Transfusion 2008;48:373-381

MDx BioRobot (Qiagen) n=1869
Bristol, UK: Finning K et al. BMJ 2008;336:816-8

QIAamp DSP Virus Kit (Qiagen) n=1022
Chemagen Separation Module 1 (Chemagen, Baesweiler, Germany)
Göttingen, Germany: Müller SP et al. Transfusion (in press)
## Automated DNA-Extraction Methods SAFE-NoE survey 2006

<table>
<thead>
<tr>
<th>Model</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Magna Pure</td>
<td>Roche</td>
</tr>
<tr>
<td>1 Magna Pure Compact</td>
<td>Roche</td>
</tr>
<tr>
<td>1 ABI 6100</td>
<td>ABI</td>
</tr>
<tr>
<td>1 Kingfisher</td>
<td>Thermo Electron</td>
</tr>
<tr>
<td>1 Separation Module 1</td>
<td>Chemagen</td>
</tr>
<tr>
<td>1 Tip Extractor (in-house)</td>
<td>Tecan</td>
</tr>
<tr>
<td>3 Biorobot MDx</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>1 Biorobot M48</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>1 EZ1</td>
<td>QIAGEN</td>
</tr>
</tbody>
</table>
The German feasibility study on fetal genotyping for RhD with maternal plasma

Four aims of this study

- Determine sensitivity, specificity, accuracy, feasibility of 1 manual and 1 automated DNA extraction procedure for testing the fetal D-status from maternal plasma

- Determine sensitivity and specificity of serologic D-determination in newborns

- Determine percentage of *RHD* positive women in D-negative pregnant women

- Determine percentage of *RHD* positive newborn among D-negative newborns
Study design

Non-invasive determination of fetal *RHD*-status from maternal plasma during pregnancy in at least 1000 cases

Comparison with D-status from the newborn, determined with serology

The serological results were obtained from maternal documents “Mutterpass” which comprise the newborn’s D-status and the documentation of Rh prophylaxis

If discrepancies were observed, final conclusions were made from repeat tests from a 2nd aliquot and buccal swap analysis of the newborn
Checklist for clinical studies

Target population defined?

Sample size determined according to prevalence/ incidence of marker?

Reference defined (cell culture, questionnaire, clinical report)?

Sample transport conditions defined?

Sample handling and storage protocol defined?

Cross-Check with clinicians positive?

Freezing capacity checked? Temperature monitored and prepared for failure of the freezer?
Topics for ethical approval

Aim of the study
Current gold standard, scientific background
Study-protocol
Recruitment of study persons, inclusion, exclusion criteria
Group-size estimate (Statistics)
Protection of Material and Data
Literature
Information leaflet
Consent leaflet
Sample collection

Network of 173 gynaecologists all over Germany

15 ml EDTA blood from each pregnant woman

Ethical approval and informed consent from all participants
Methods

- 15ml EDTA-anticoagulated blood/pregnant women
- Transport at room temperature 0 – 8 days (median 2 days)
- 10min/2,700g, plasma re-centrifugation 45min/12,500g
- Storage of plasma and buffy coat at -80°C
- ABO-, Rh-, Kell-status from maternal red blood cells
- D-status via indirect anti-globulin test

2x 0.5ml: QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) spin-protocol, “spin columns” (Legler et al., Prenat Diagn 2007;27:824-29)

1x 1.0ml chemagic Magnetic Separation Module 1 (Chemagen, Baesweiler, Germany) “magnetic tips”

RHD exons 5/7
2 replicates/sample

Control: β-globin PCR
Chemagen Magnetic Separation
Module 1

(Chemagen, Baesweiler, Germany)
Magnetic tip

cover

collect beads
large volume

remove beads

Transfer to small volume

wash

Remove magnetic tip

Beads drop off

Remove cover

Remove magnetic tip

cover

Beads drop off

Remove cover
First evaluation of cff extraction using magnetic particles

<table>
<thead>
<tr>
<th>Method</th>
<th>Manufacturer</th>
<th>Volume, extracted (V_ex)</th>
<th>Volume/PCR</th>
<th>Positive reaction (+) according to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tip-Extraktion</td>
<td></td>
<td>500</td>
<td>500</td>
<td>+  +  +  +  +  +  +  -</td>
</tr>
<tr>
<td>Ultransense Kit</td>
<td>Qiagen</td>
<td>1000</td>
<td>33</td>
<td>+  +  +  -  -  -  -  n.t.</td>
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<tr>
<td>QIAamp DNA Blood Midi Kit</td>
<td>Qiagen</td>
<td>2000</td>
<td>17</td>
<td>+  +  +  +  -  -  -  n.t.</td>
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<tr>
<td>Mini-Kit, 20 µl elution</td>
<td>Qiagen</td>
<td>200</td>
<td>11</td>
<td>+  +  +  +  -  -  -  n.t.</td>
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<tr>
<td>QIAamp viral RNA</td>
<td>Qiagen</td>
<td>140</td>
<td>14</td>
<td>+  +  +  +  -  -  -  n.t.</td>
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<tr>
<td>Genomix</td>
<td>Talent</td>
<td>2400</td>
<td>86</td>
<td>+  +  -  -  -  -  -  n.t.</td>
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<tr>
<td>Seek viral DNA</td>
<td>Talent</td>
<td>200</td>
<td>2</td>
<td>+  +  -  -  -  -  -  n.t.</td>
</tr>
<tr>
<td>Seek viral RNA</td>
<td>Talent</td>
<td>200</td>
<td>6</td>
<td>+  -  -  -  -  -  -  n.t.</td>
</tr>
</tbody>
</table>

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Real-time PCR

- **RHD** exons 5 (\(D\psi\) neg.) and 7 (one exon had to be positive)
- 2 Replicates/sample:
  2/2 positive/negative → D-positive/negative
  1/2 replicates positive → Repeat
  1/4 positive → D-negative
  2-3/4 positive → D-positive
- Positive/negative controls
- Genomic DNA-standard, calculation of quantitative data: imported standard curve (Zimmermann et al., Clin. Chem. 2005, 51 (9); 1598-1604)
- External control: **β-Globin**

If quantity of D-positive DNA was more than 20% of the quantity of **β-Globin** positive DNA, maternal buffy coat was tested for the presence of **RHD** and **RHD** sequencing performed.

*Applied Biosystems, Foster City, USA*
Subjects

1113 women, gestational week 6-32 (median 25), all typed as serologically D-negative according to German guidelines (at least 2 monoclonal IgM anti-D’s which do not detect DVI, test on auto-agglutination)

5 identified as carriers of \( RHD \text{ ex}5/7 \):
- weak D type 1
- \( RHD (M295I) \), DEL, C+c+E-e+
- \( RHD \) (93-94insT), C+c+E-e+, 2x
- \( RHD \) without apparent polymorphism ex1-10 (\( RHD \) unexpressed)

16 hemolytic, 7 serum samples

1084 apparent \( RHD \) ex5/7 deletions, 1 \( RHD\Psi \)

1022 (94.2%) postnatal cord blood serology \( \leftrightarrow \) real-time PCR results
## Results PCR - Serology

<table>
<thead>
<tr>
<th></th>
<th>Serology D-positive</th>
<th>Serology D-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time PCR</td>
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<td></td>
</tr>
<tr>
<td><em>RHD</em>-positive</td>
<td>657</td>
<td>10</td>
</tr>
<tr>
<td>(manual and/or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>automated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real-time PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>RHD</em>-negative</td>
<td>2</td>
<td>353</td>
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<tr>
<td>(manual and/or</td>
<td></td>
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</tr>
<tr>
<td>automated)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Why is this study unique?

• Serology was not the only reference

• If a buccal swab was required, this was obtained by informed consent (former studies tested plasma from antibody screening tubes)
# Results

1022 samples

In 12 cases, real-time PCR results were discrepant from serology

<table>
<thead>
<tr>
<th>Sample</th>
<th>False Negative</th>
<th>False Positive</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>spin columns magnetic tips</td>
<td>week 25, transport 6 days, repeat tests* (14pg/ml) and RHD sequencing pos.†</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>spin columns</td>
<td>week 22, repeat test and magnetic tips pos., but yield of fetal DNA below average</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>serology</td>
<td>RHD sequencing pos. 1 normal RHD, repeat serology pos. 2 weak D type 2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>spin columns magnetic tips</td>
<td>repeat tests and RHD sequencing neg.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>magnetic tips</td>
<td>repeat tests neg.</td>
<td></td>
</tr>
</tbody>
</table>

*repeat test from a fresh sample aliquot; †Sequencing from newborns buccal swab-DNA

662 (64.8%) D-positive 360 (35.2%) D-negative
Antenatal real-time PCR is as sensitive as postnatal serology for the determination of the child’s D-status, serology is more specific.
Fetal DNA Yield (RHD exon 7)
all D-pos samples

DSP Virus Kit: 6 – 6973pg/ml, median 101pg/ml
Chemagen Magn. Sep. Mod. 1: 43 – 34.418pg/ml, median 703pg/ml
Summary

6 (0.55%) RHD positive women in 1090 D-negative pregnant women. 1 woman with weak D type 1 (0.1%) was falsely determined as D-negative with serology. Only 1 RHDψ in this study group.

3 (0.8%) RHD positive newborn in 363 serologically D-negative newborn, 2 cases (0.5%) with weak D type 2 and 1 mistyping were false negative in serology.

False negative determination of fetal D-status with rt-PCR may occur. 1 case (0.15%) with CHEMAGEN, 2 (0.30%) cases with QIAGEN. Sample transport time might be crucial. Duplication of work and costs (2 extraction, 4 PCR) would have avoided those false negative cases. A universal marker, which confirms presence of fetal DNA, would increase reliability.

Compared with QIAGEN lower specificity is obtained when DNA is extracted with CHEMAGEN, whereas higher yields of fetal DNA and higher sensitivity are achieved.
Conclusions

This large prospective clinical trial indicates that routine non-invasive determination of fetal \textit{RHD}-status is \textit{feasible}.

In case of a D+ fetus the \textit{prenatal determination} of the fetal D-status would allow \textit{immediate anti-D prophylaxis} after birth instead of waiting for a blood group determination from cord blood.

The implementation of this procedure would allow to \textit{safe anti-D immunoglobulin} in cases, where this blood product is not indicated. Thus, pregnancies, that occur in D-negative women, could be treated in a \textit{safer and more effective} way.

A commercial fetal RhD genotyping Kit with CE mark is available since July 2007 from Institut de Biotechnologies Jacques Boy, Reims, France.
SAFE: *RHD NI PD Standardisation workflow*

1. A reference measurement procedure was determined
2. Diagnostic sensitivity and specificity were determined for the most promising technology platforms in large-scale single-centre studies
3. A reference material of a higher order for the determination of the analytical sensitivity was agreed in a workshop
4. A Quality Assurance scheme for gauging levels of repeatability, consistency and uniformity was established
5. Agreement was obtained in a discussion workshop that the internal control is not mandatory for RHD NIPD screening.
RhD/ SRY Plasma Sensitivity Standard for NIPD
(slide provided by Evelyn Tait, University of Aberdeen)

Heterozygous, RhD positive male donor *mimics* fetal plasma

RhD negative, female donor *mimics* maternal plasma

Ultrasound profile at 13 weeks
RhD/SRY Plasma Sensitivity Standard for NI PD

RhD/SRY Plasma Standard 5 (1-in-8) appeared to function as a reliable sensitivity control for both RhD and fetal Sexing across SAFE laboratories.

Standard 5" was the recommended "dilution" for a Sensitivity Control to be prepared on a large scale by the National Institute for Biological Standards and Controls (NIBSC, South Mimms UK) (~2-3000 vials) and made available to the international scientific community involved in NIPD.
The International Blood Group Reference Laboratory (IBGRL)* in Bristol, UK, (http://ibgrl.blood.co.uk/) organizes RHD NIPD Workshops on behalf of the International Society for Blood Transfusion (ISPD) and the International Council for Standardization in Haematology (ICSH) every two years since 2004 (2006, 2008). Voluntary testing for other bloodgroups (RhC/c, Kell) is possible.

*IBGRL is partner of SAFE.
Possibilities for internal controls

• Y-Chromosome (\textit{SRY} rather than \textit{DYS14})

• Ins/Del Polymorphisms

• placental m-RNA

• Methylation dependent PCR/RFLP
Which internal control material is acceptable?

• None of the universal foetal DNA marker are currently available for large scale routine applications

• Plasmid DNA or packed (armored) DNA as internal control is an option if no universal fetal marker is available. It is even possible to test without internal control in screening applications.
Which **run control/validation material** is acceptable?

- Plasma Pools from maternal plasma
- Donor plasma (artificial mixtures)
- Placenta perfusion material
- DNA from *RHD* variants diluted in D-negative plasma
SAFE: *RHD NI PD Standardisation workflow*

1. A reference measurement procedure was determined
2. Diagnostic sensitivity and specificity were determined for the most promising technology platforms in large-scale single-centre studies
3. A reference material of a higher order for the determination of the analytical sensitivity was agreed in a workshop
4. A Quality Assurance scheme for gauging levels of repeatability, consistency and uniformity was established
5. Agreement was obtained in a discussion workshop that the internal control is not mandatory for RHD NIPD screening.
Conclusions

1. The SAFE DNA extraction protocol for the QIAamp DSP Virus Kit was selected as reference measurement procedure.

2. Pools of plasma from pregnant women should be applied as run and validation material, however NIBSC will provide a reference material from donor plasma for comparison.

3. Diagnostic sensitivity and specificity has been determined for MagnaPure, MDx, Chemagen and QIAGEN Virus Kit.

4. A Quality Assurance scheme has been established for RHD NIPD by the IBGRL/ISBT.

5. The consensus process was facilitated by travel grants, wet workshop grants, confidential agreements, and biobanking grants from the European Commission.
NI PD Application beyond blood groups


- Detection of paternal alleles with and without enrichment of smaller DNA fragments
- β-Thalassemia: β-globin gene (200 mutations)
- Myoton Dystrophy: DMPK Gen
- Achondroplasie: FGFR3 Gen
- Cystic fibrosis: Q890X (1000 Mutationen)
- hemoglobin E/β-Thalassemia: Hämoglobin E Gen

New Technologies: mass spectometry of PCR products (MALDI-TOF, details: Sequenom Inc.)
SAFE - Achievements

NIPD for single gene disorders

- Established a growing biobank of samples from pregnancies affected with aneuploidy and single gene disorders to help expedite implementation as the technology improves.

- Extensive work on Thalassaemia
  - Increasing number of NIPD-assays of mutations for β-Thalassemia
  - NIPD assays being developed for polymorphisms linked to beta-globin gene to detect the paternal allele

- NIPD for achondroplasia being established
### Recessive disorders: Enrichment of shorter (fetal) DNA fragments

Hromadnikova et al. DNA and cell biology 2006;25:635-640

<table>
<thead>
<tr>
<th>Fragment-length after gel-electrophoresis (base-pair, bp)</th>
<th>n=11</th>
<th>Uncut</th>
<th>100-300</th>
<th>300-500</th>
<th>500-700</th>
<th>700-900</th>
<th>&gt;900bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>% fetal DNA (median)</td>
<td>0,32</td>
<td>1,04</td>
<td>1,0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>enrichment (median)</td>
<td>4,2</td>
<td>2,5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

05.06.08
Bisulfit-treatment and methylation -specific PCR/ RFLP
Chim SSC et al. PNAS 2005;102:14753-14758

<table>
<thead>
<tr>
<th>Case</th>
<th>Maternal</th>
<th>Placenta</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>258</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>272</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>300</td>
<td>A</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>310</td>
<td>A</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>331</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>X</td>
<td>AC</td>
<td>CC</td>
<td>AC/CC</td>
</tr>
<tr>
<td>427</td>
<td>AC</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>454</td>
<td>AC</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

methylated (maternal): C→C

Unmethylated (Placenta): C→U

Unmethylated (Placenta): C→U
Summary

Available

• Fetal RhD determination (Göttingen, Bristol, Amsterdam)
• Determination of the fetal sex (Bristol, London, Amsterdam)
• RhCE, (Kell), C, c, E (Bristol, Amsterdam, Göttingen)
• HPA-1 (Bristol, Amsterdam)

Successful proof-of-principle studies

• Single gene disorders
• Chromosome disorders

Further Research required (proteomics, transcriptomics)

• preeclampsia
• Intrauterine growth retardation
• Preterm labour
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Peter Sedelmayr, Neil Avent

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Thank you for your attention!