Cell free fetal DNA in maternal blood

From science to a nationwide screening program

Ellen van der Schoot, Sanquin

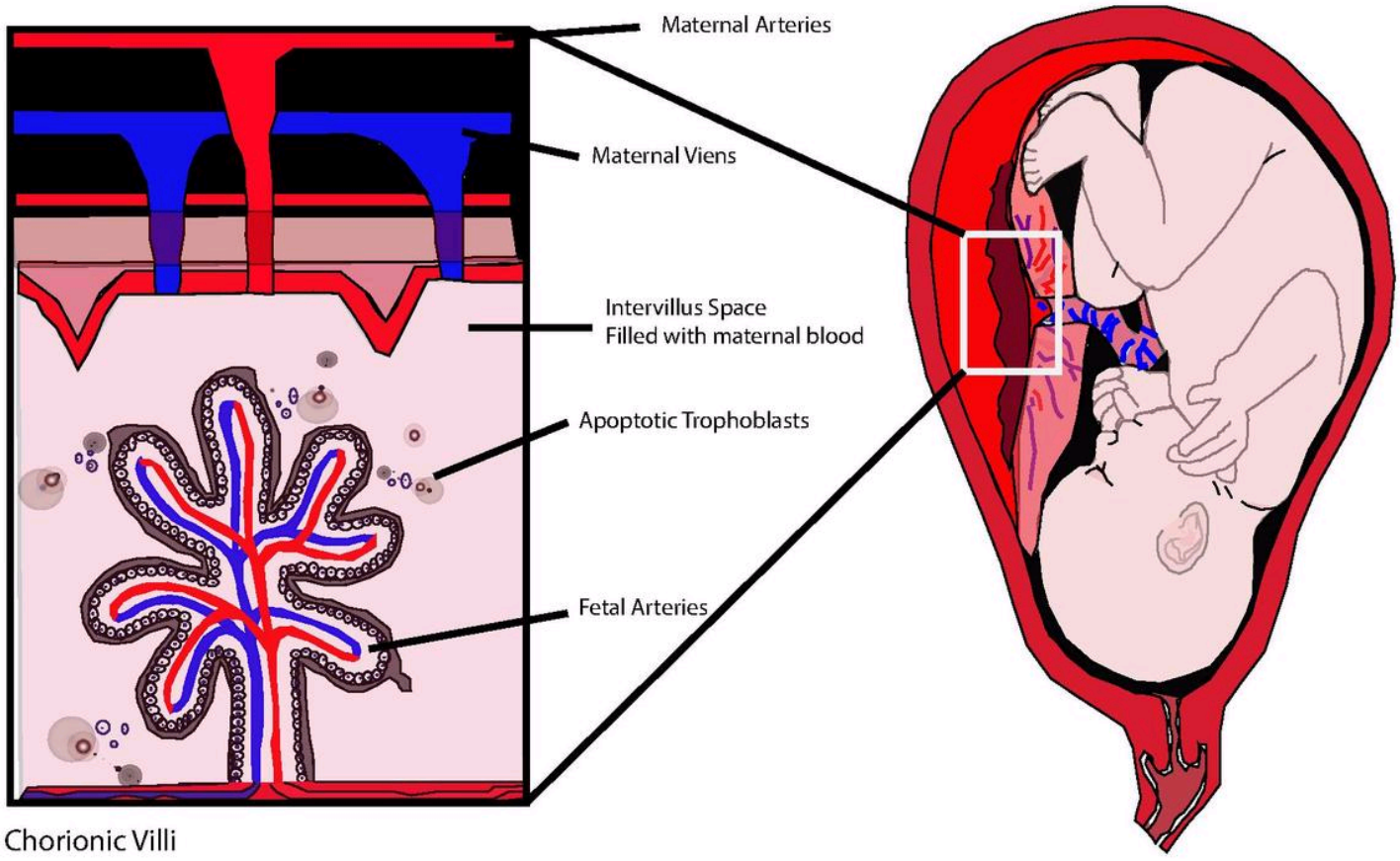
Prenatal Diagnostics

- **Invasive :**
  - Fetal DNA obtained from amniocytes
  - Fetal DNA obtained from chorionvilli

- **Non-invasive:**
  - Fetal DNA or RNA from circulating fetal cells
  - ➢ Fetal DNA (or RNA) from maternal plasma

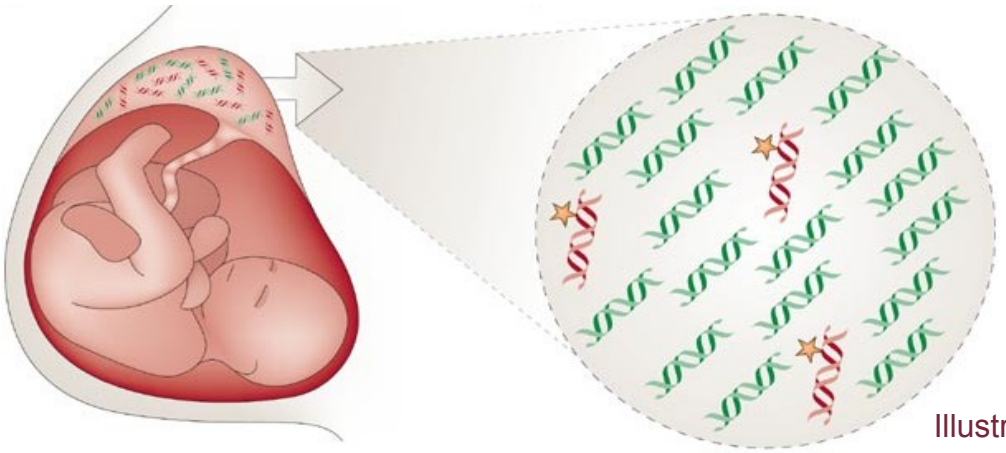
    YM Lo et al., Lancet 1997;350:485-7

Source of fetal DNA: apoptotic syncitiotrophoblast



Cell-free fetal DNA in maternal plasma

placenta → plasma of pregnant woman



Mixture of DNA from mother and DNA from fetus

Illustration: Lo, 2007

Excess of maternal cell-free DNA:

- 11-17 weeks: 3% fetal DNA (range: 0,4% - 12%)
- 37-43 weeks: 6% fetal DNA (range: 2,3% - 11,4%)

Cell free DNA is derived from apoptotic cells

- Present in plasma as nucleosomes
- Majority of cell free fetal DNA < 143 bp
- Cell free maternal DNA: majority >143bp:
  - Mainly derived from maternal leukocytes
  - Increased in various conditions: e.g. sepsis, autoimmune diseases, pregnancy

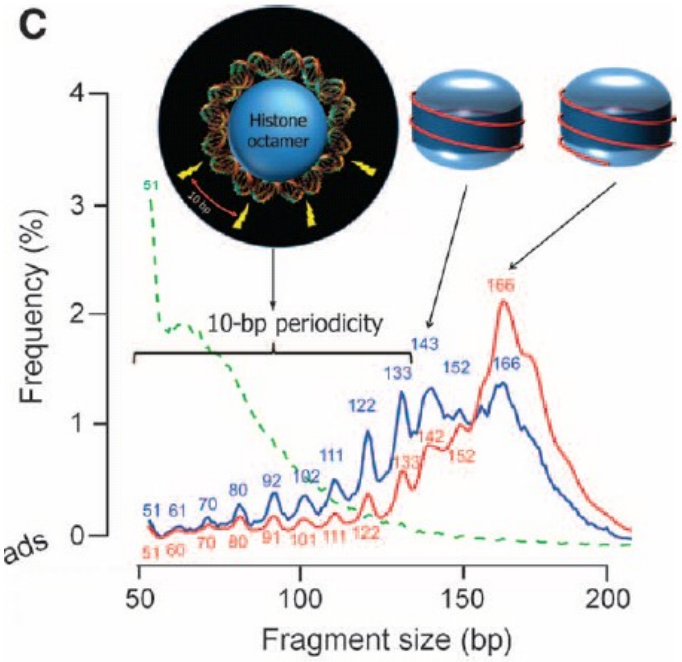


Illustration Lo 2010

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Concentration of cell free fetal DNA

- Earliest presence: 5 weeks of gestation
- Very low concentration of fetal DNA in maternal plasma
  - 16th week: 25 genome equivalents/mL of plasma (range 3-70 geq/mL)
  - 30th week: 290 genome equivalents/mL of plasma (range 50-1000 geq/mL)
- Half-life: 15 minutes

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SAFE Network of Excellence -

- Large European Consortium (25 laboratories, 2005-2010)
  - Non Invasive Prenatal Diagnostics
- First clinical applications have been developed in this network
- Standardized fetal DNA isolation procedure from plasma,
  - Plasma standards (NIBSC)
  - Proficiency testing

safenetwork

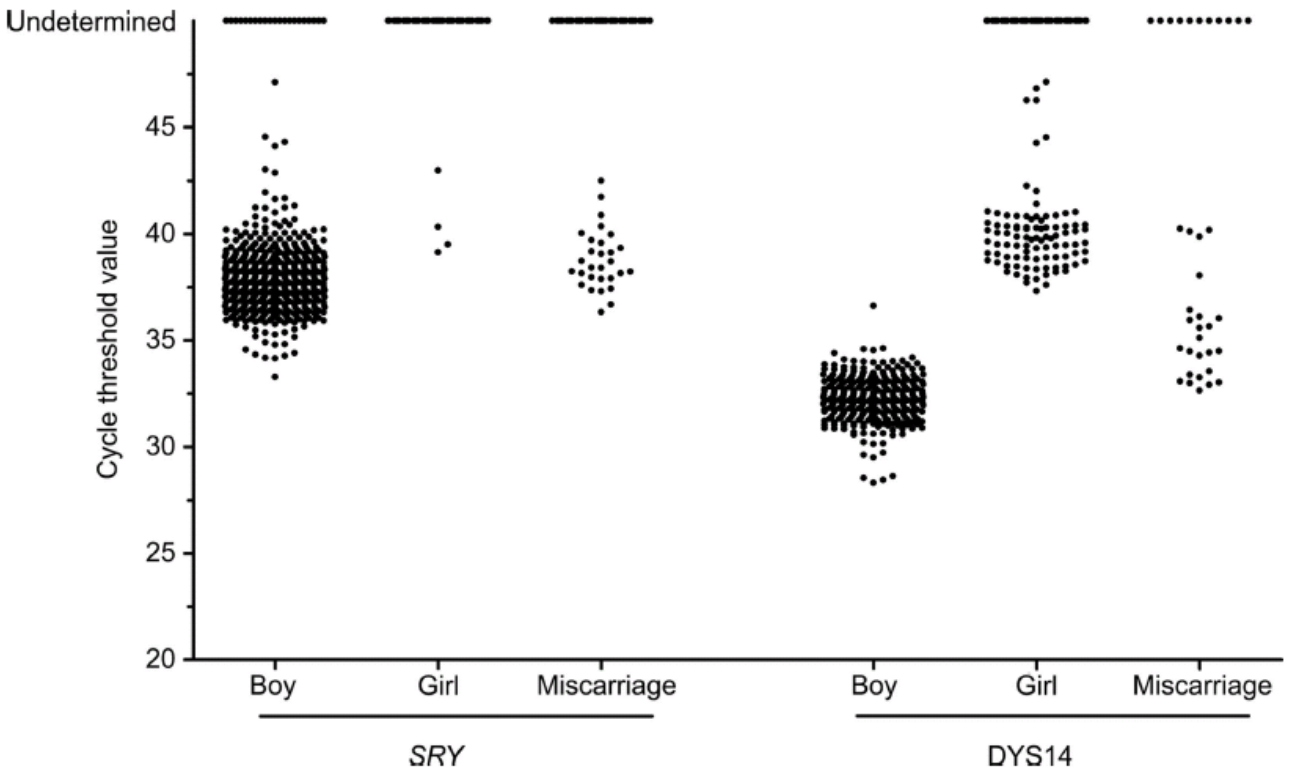
Applications of cell free DNA

- Fetal sex determination
- Fetal genotyping for bloodgroups
- Diagnosis of monogenic inherited disorders
  - E.g. Thalassemia, Sickle cell anaemia
- Since Next generation Sequencing:
  - **Aneuploidies,** e.g. Trisomy 21
  - Inherited diseases

Presence of paternally derived DNA sequences

Real time PCR

Reliability of fetal sex determination : 100%



N=200

Scheffer et al.. Obstet Gynecol 2010

Low concentration ccfDNA : need for fetal identifier

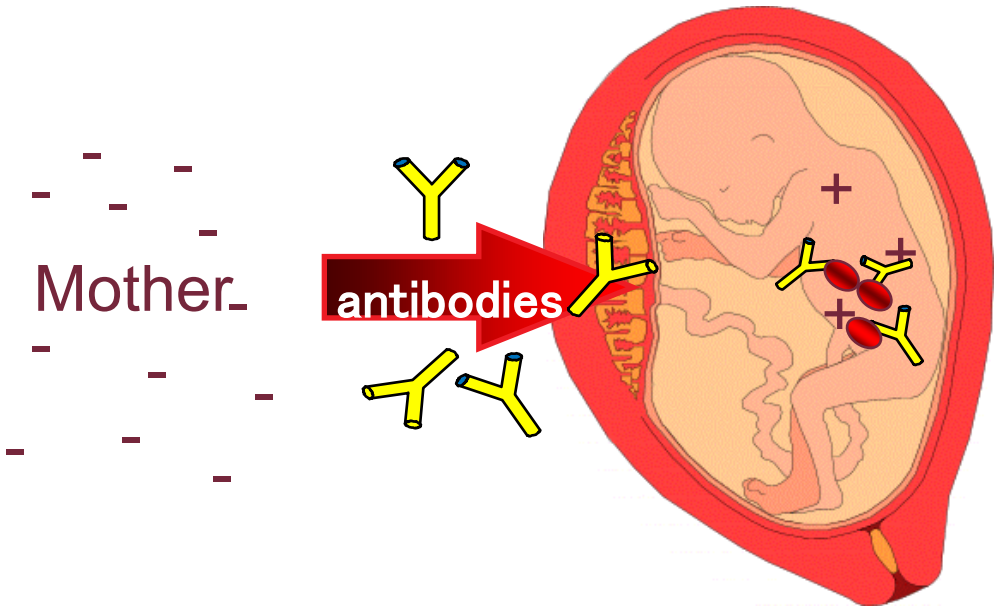
- **Polymorphisms** : Mother negative , Father (homozygous) positive
  - Panel of SNPs
  - Panel of Ins/Del polymorphisms
  - Panel of Copy Number Variation (0,1,2)

    - Laborious, large panel

- **Universal fetal identifier** : Mother negative, fetus positive
  - Epigenetic marker: Hypermethylated RASSF1a

    - Not very reproducible, lower sensitivity

Fetal blood group typing



1) Red cells: HDFN

Hemolytic Disease of Fetus/newborn
⇒ Kernicterus

2) Platelets: FNAITP

Fetal/neonatal alloimmune thrombocytopenia
=> Intracranial hemorrhages

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Fetal blood group typing

- Diagnostics:
  - In alloimmunized women to start timely treatment
- Screening
  - To guide anti-D immunoprophylaxis

Nation-wide fetal RHD genotyping introduced July2011 in the Netherlands

To prevent immunization during pregnancy ALL D-negative pregnant women get anti-D immunoglobulin

Anti-D Ig:

- Bloodproduct
- Volunteer, hyperimmunized donors
- World wide shortage
- Costs

→ 40% of D-negative women are carrying D negative fetuses

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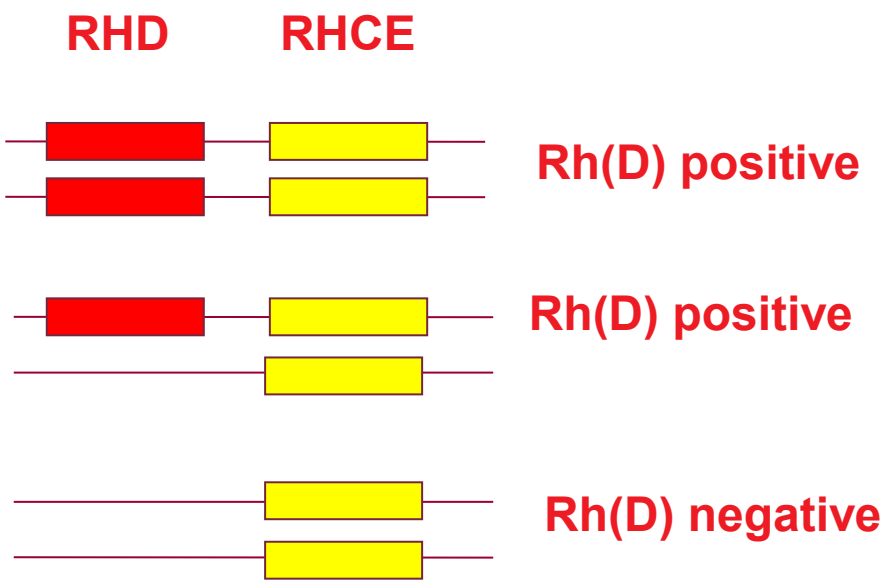
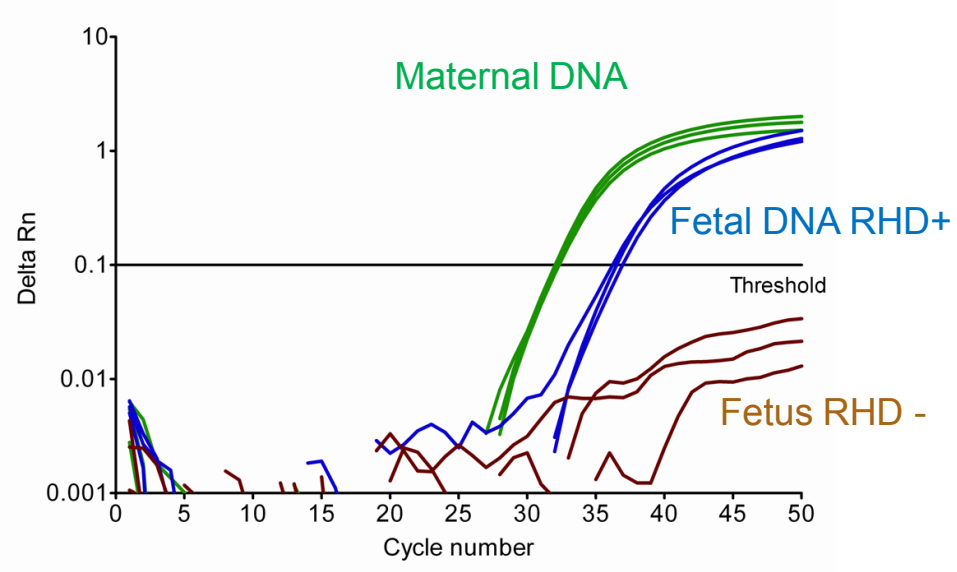
Rh-system:

- Coded by 2 homologous genes: RHD en RHCE
- D-negativity in Caucasians is caused by deletion of RHD gene





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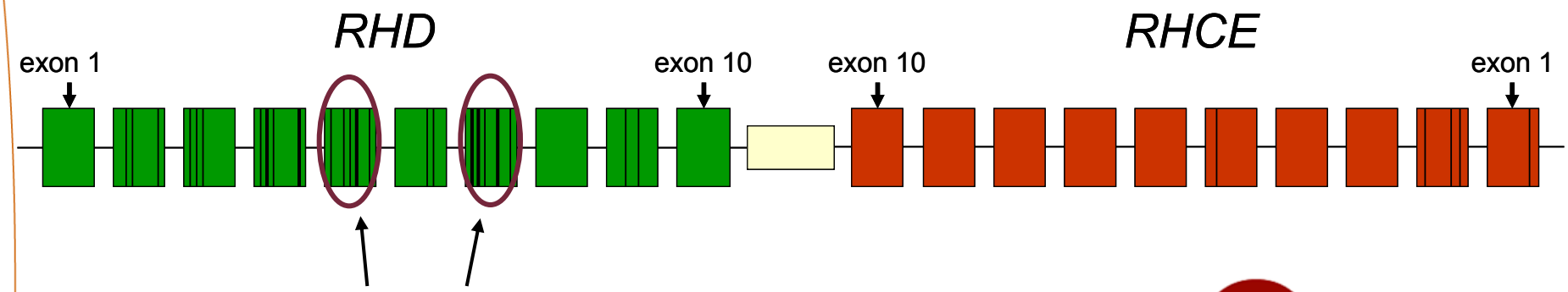
Design fetal *RHD* typing



***RHD*-PCR Multiplex**

exon 5: not amplified in majority of *RHD* variants

in Caucasians: RHD*DVI

in Blacks: RHD*Ψ; RHD*01N.06 and RHD*03N.01 (r's)

exon 7: present in most *RHD* variants

Scoring algorhitm:

Ct <40 is positive (Ct < 20 is artefact)

Exon is positive if ≤ 1 replicate is negative

Sample is positive if at least 1 exon is positive

Fully automated approach

Centralized at one laboratory (Sanquin, Amsterdam)

7-8 cc EDTA anti-coagulated blood

DNA isolation from **1 ml of plasma**

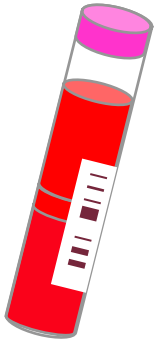
- Eluate 50 µl

Robotic workstation for PCR setup

- RQ-PCR **in triplicate** (15 µL input/well), 50 cycli
- NO fetal identifier, NO total DNA control

Real-time PCR

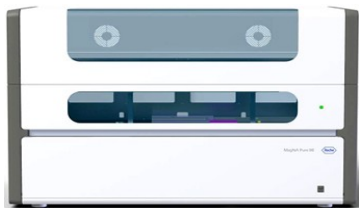
– 25 µL PCR



Electronic Result

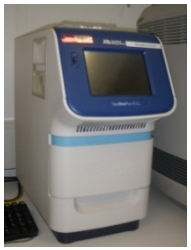
Plasma separation → **Purification RNA/DNA** → **PCR Setup** → **Amplification Detection** → **Report**

Xyril

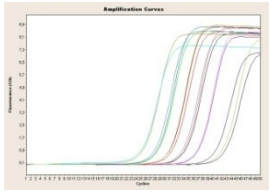


MagnaPure 96

Xyril



StepOnePlus



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Aim of the study

• Evaluate the sensitivity and specificity of the new fetal *RHD* screening program introduced in July 2011

• Prerequisite of the Dutch anti-D prophylaxis programme:

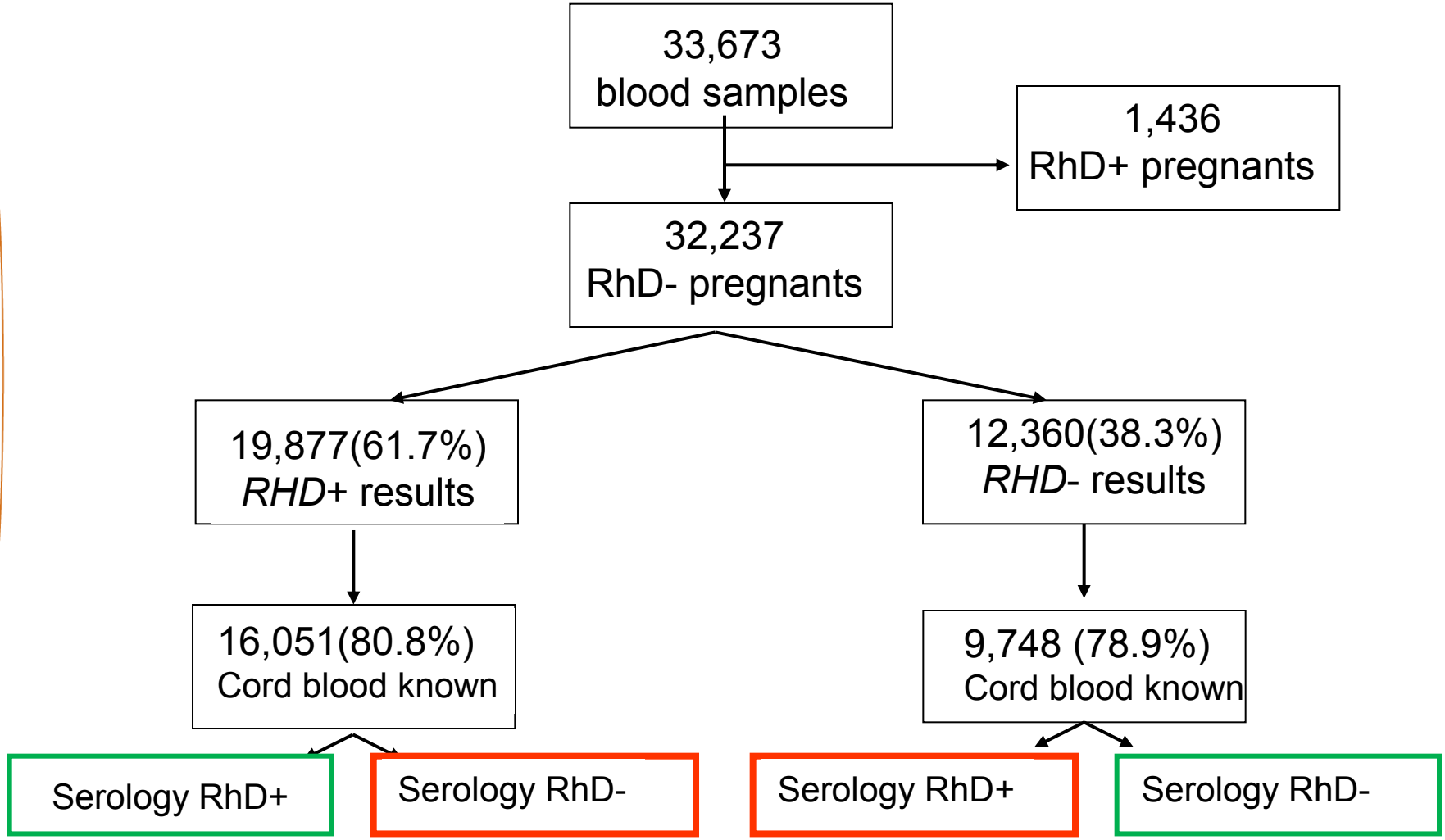
Sensitivity : <0.25% False negative/ all pregnancies

=estimated false negative rate cord blood serology (Koelewijn et al. 2008, Legler et al. 2009)

Specificity : no fixed target

In the first 15 months all cord blood samples were sent to Sanquin

Results of the first 15 months



False positive?

False negative

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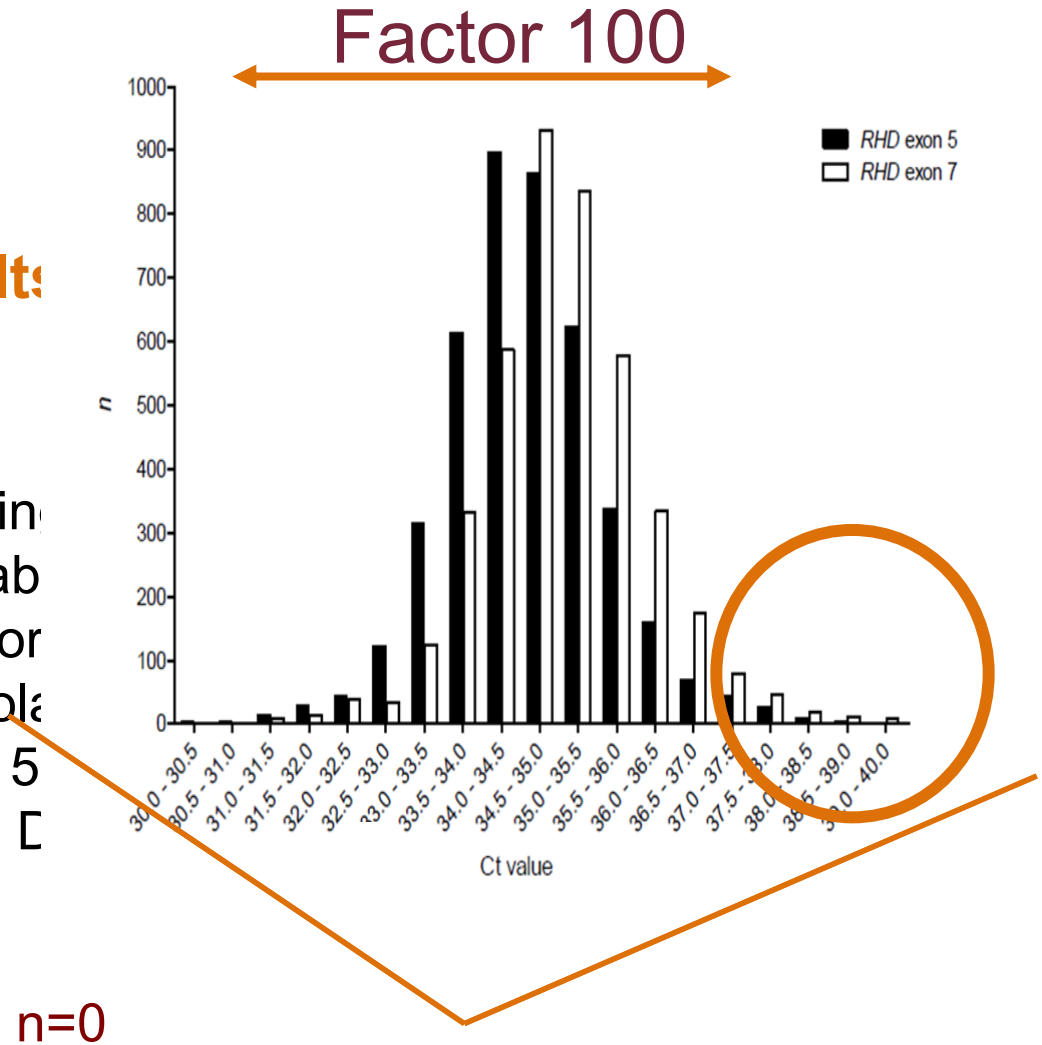
8 false negative results



Repeat testing

- DNA fingerprintin
blood/buccal swab
- *RHD*-PCR on cor
- Manual DNA isol
- Monoplex exon 5
and mRASSF1a, D

Results

- Sample mix up: n=0
- Fetal DNA concentration low: n=6
- Technical failure or putative technical failure: n=2

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Evaluation of false positive results (0.88%)

PCR algorithm is aimed to prevent false negative results

I. Aspecific amplifications are scored positive : 0.47%

II. Fetal variants are scored positive by PCR, but might be missed by serology (0.09%) or do not lead to RhD-expression : 0.18%

III. All D-negative mothers carrying non-functional *RHD* alleles or variant RHD alleles are scored positive : 0.22%

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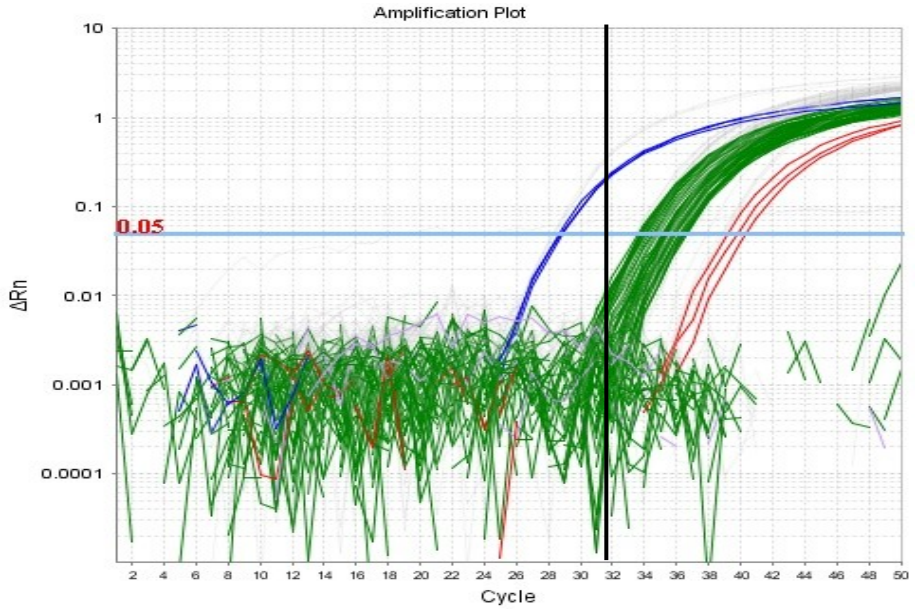
III. Maternal RHD-variants



Amplification of maternal *RHD* DNA hides fetal DNA

All maternal variants have been analyzed

=> Known and new RHD variant genes

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Conclusions on fetal DNA typing

- High level of reliability of fetal *RHD* typing
  - False negativity: 0.03% (95% CI 0.01 – 0.05%)
  - False positivity: 0.88% (95% CI 0.77 – 1.00%)

- If fetal *RHD* typing is performed around week 27 of pregnancy, cord blood RhD serology can be safely omitted

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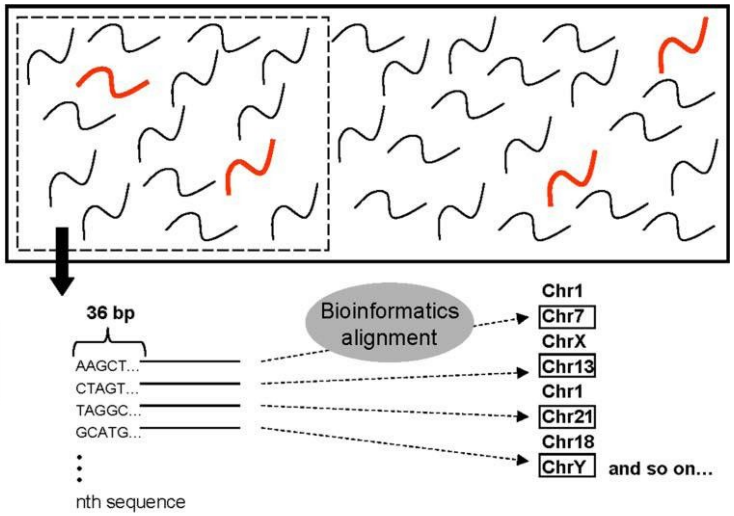
Cell free fetal DNA for genotyping fetus

- Genotyping assays can detect all paternally inherited mutations
- By “counting” or “allelic ratio” also presence of maternal alleles can be shown
- But MOST IMPORTANTLY:
  - Next generation Sequencing opened the possibility of NIPD for aneuploidies
  - Whole genome sequencing of fetus (Lo YM et al. Sci Transl Med. 2010)

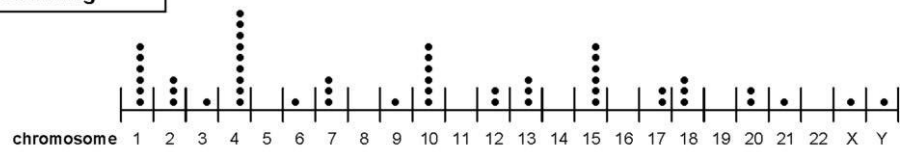
Trisomy testing by NGS

DNA fragments in maternal plasma

Sequence and align



Sequence counting

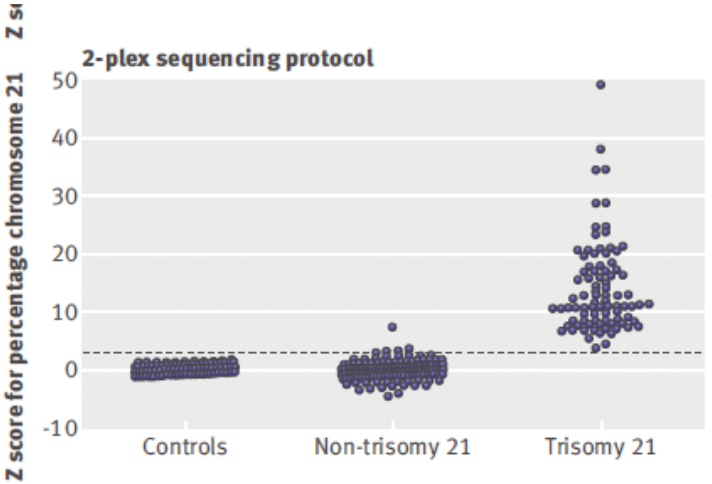


% representation of unique sequences mapped to a chromosome

$$\%\ \text{chrN} = \frac{\text{Unique count for chrN}}{\text{Total unique count}}$$

Disease status determination

$$\text{chrN z-score for test sample} = \frac{\%\ \text{chrN}_{\text{sample}} - \text{mean}\ \%\ \text{chrN}_{\text{reference}}}{\text{S.D.}\ \%\ \text{chrN}_{\text{reference}}}$$



Chiu et al. BMJ 2010

Trisomy testing is available in US, Germany, Belgium

- Sensitivity and Specificity > 99% in high risk population
- In the US commercial assays available:
-

able in the United States.*

Test	Company	U.S. Launch Date	Cost	Sensitivity	Specificity
			$	percent	
Verifi	Verinata	February 2012	1,200 (cost sharing capped at 200)	Trisomy 21, >99.9; trisomy 18, 97.4; trisomy 13, 87.5	Trisomy 21, 99.8; trisomy 18, 99.6; trisomy 13, >99.9
MaterniT21	Sequenom	October 2011	2,762 (cost sharing capped at 235)	Trisomy 21, 99.1; trisomy 18, >99.9; trisomy 13, 91.7	Trisomy 21, 99.9; trisomy 18, 99.6; trisomy 13, 99.7
Harmony	Ariosa	May 2012	795	Trisomy 21, >99.9; trisomy 18, 98.1; trisomy 13, 80.0	Trisomy 21, >99.0; trisomy 18, >99.0; trisomy 13, >99.0
Panorama	Natera	December 2012†	1,495	Trisomy 21, 100; trisomy 18, 100; trisomy 13, 100	Trisomy 21, 100; trisomy 18, 100; trisomy 13, 100

N ENGL J MED 369;6 NEJM.ORG AUGUST 8, 2013

The Netherlands: NITRO consortium

- Trisomy screening is regulated by “Wet op Bevolkingsonderzoek” WBO
- Study in high risk pregnancies
  - Patients with positive “Combination test” => Will be offered NGS

Expectation:

Implementation for high risk pregnancies in 2014

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Conclusions

- Cell free fetal DNA is present in variable, low concentrations in maternal plasma (10-1000 geq/ml) in the background of maternal DNA (2-10%)
- Highly stable, rapidly cleared after birth
- Derived from apoptotic placental cells
- Can reliably used for genotyping of fetus
- In the near future NIPD will completely replace invasive prenatal diagnostics