Fetal DNA in maternal plasma, a new source for prenatal genotyping

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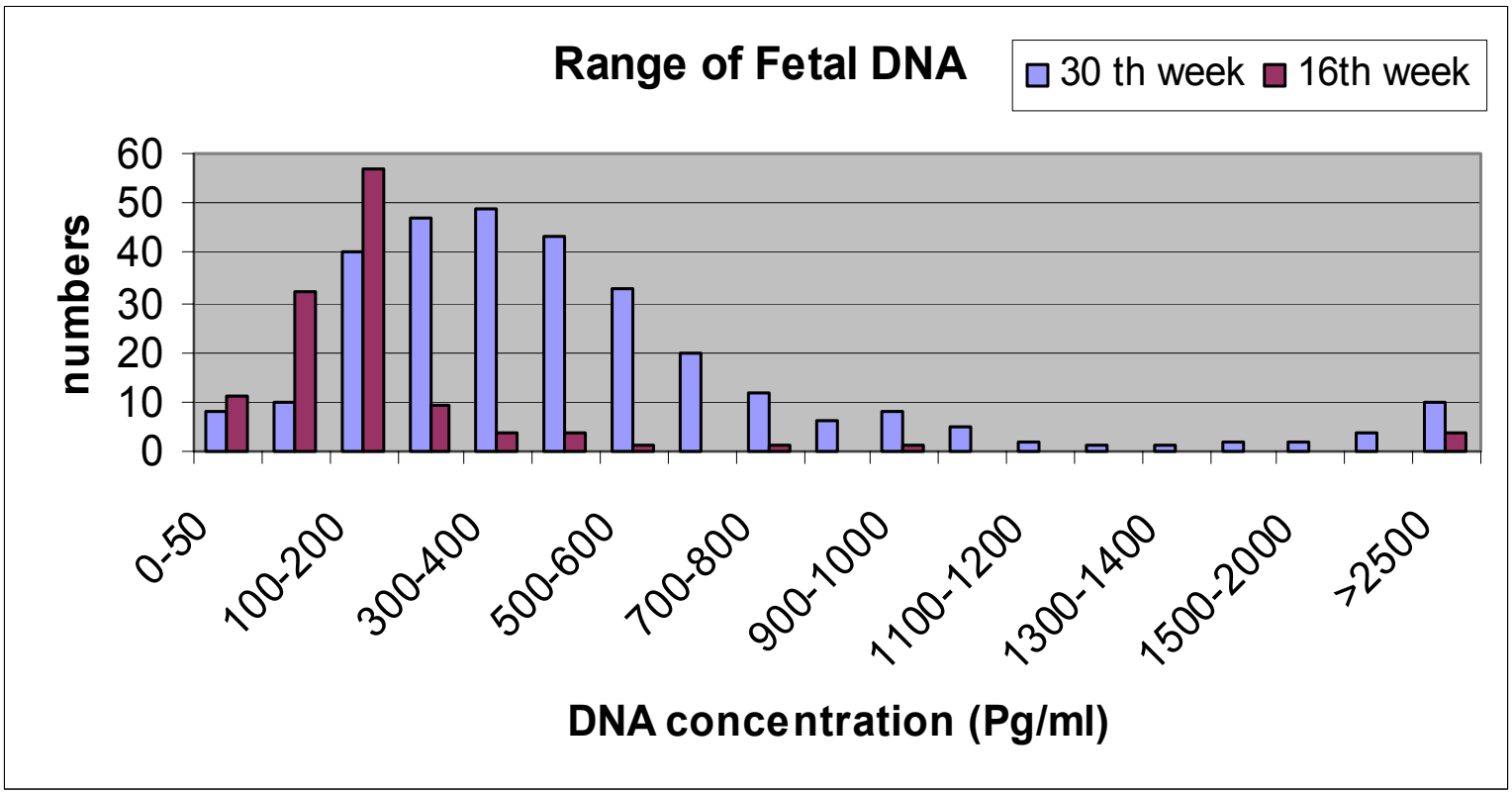
Plasma DNA

- Present in very small amounts in plasma of normal individuals. *Cell free, impossible to spin down*
- Increased in patients with cancer, tumor-associated DNA mutations are present in plasma DNA
- Placenta can be seen as a pseudomalignant tissue => Lo et al. hypothesized that placental derived fetal DNA is present in plasma
- First demonstration: Lo et al. Lancet 1997; 350: 485-487

Fetal DNA concentration

30th week: n = 299, Mean 522 pg/ml (range 20-4640) => **79** geq/ml

16th week: n = 120, Mean 149 pg/ml (range 23-952) => **23** geq/ml



Geq= genome-equivalent/ml

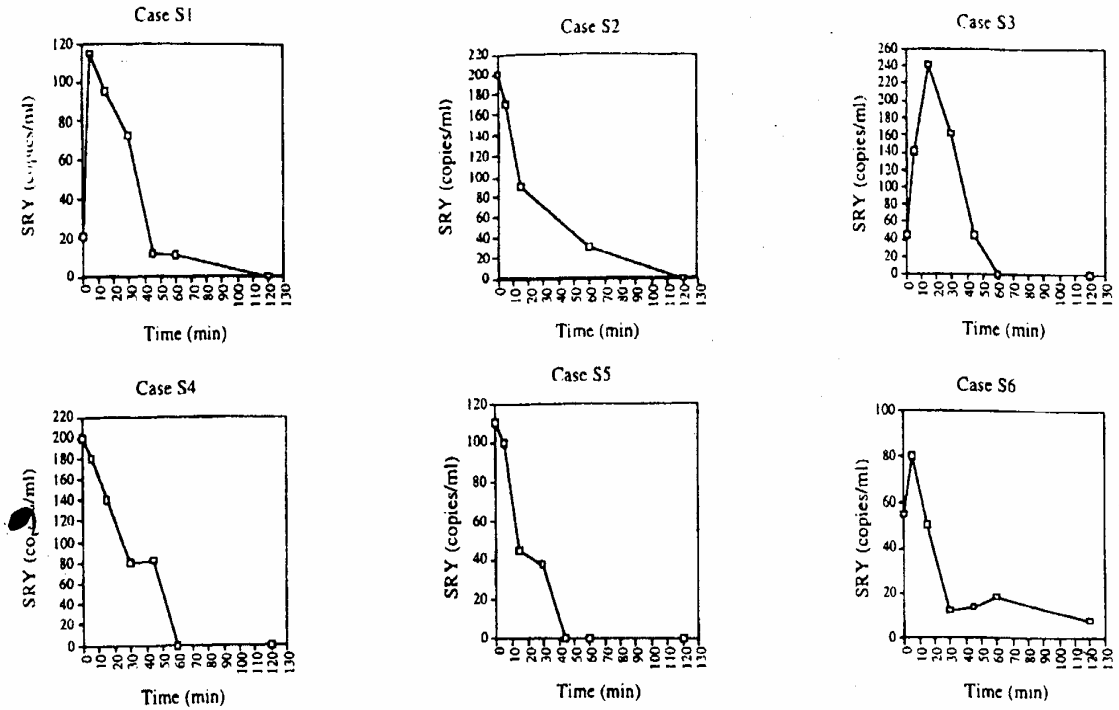
Fetal DNA in plasma

- Earliest detection of fetal DNA: 5 weeks of gestation (*Prenat Diagn 2003; 23, 1042*)

- Fetomaternal ratio in plasma*(Am J Hum Gen 1998; 62: 768)*
  - 11-17 weeks : 3,4 % (range: **0,39 - 11,9%**)
  - 37-43 weeks : 6,2 % (range: **2,33% - 11,4%**)

- Source of fetal DNA?
  - From fetal cells in the maternal circulation?
  - **From cells in the placenta**

Rapid clearance of fetal DNA from maternal plasma *(Lo et al. Am J Hum Gen 64:218-24, 1999)*



T1/2 = 16 minutes (range 4-30)

False positivity due to persistence of DNA from previous pregnancies?

- Ivernizzi et al. Hum Genet 2002; in 35/160 (**22%)** healthy women with male offspring after delivery (range 1-60 yrs) a positive Y-PCR was found

- => study on 120 women (25-75 yrs) (Rijnders et al. Clin Chem. 2004;50:679-81)
  - » 64 male offspring
  - » 13 only female offspring
  - » 43 without children

Conclusion: No persistence of fetal DNA after delivery

Blood group antagonisms

- Per definition we are looking for DNA sequences that are not present in the mother
  - => no need for purification of fetal DNA => plasma DNA is ideal source
  - => with PCR-based assays it is possible to detect a single copy of a fetal gene
- Clinically most relevant:
  - red cell antigens: **Rh(D),** Rh(c), K
  - platelet antigens: HPA-1a

Application of fetal RhD typing in plasma

- Non invasive fetal bloodgrouptyping in alloimmunized mothers
  - Positive predictive value is virtually 100%, but false negative results are encountered
    - Need for a positive control, Y-PCR in 50%

➔ To restrict antenatal immuno-prophylaxis to RhD-negative women carrying a RhD positive child

Antenatal immunoprophylaxis

- To decrease the incidence of RhD-alloimmunization, D-negative pregnant women receive anti-D IgG in the 28-32th week of pregnancy

- About 40% of these women are carrying D-negative fetuses

Aim of the study

- Development and validation of a non-invasive, high-throughput, fetal RhD genotyping assay to restrict antenatal prophylaxis
  - fully automated
  - sensitivity > 95%
  - false positive results are less cumbersome
  - assay-costs < half of the antenatal immunoprophylaxis costs

Fully automated Assay

Bar coded tubes

Centrifugation(2x)

Tecan Pipetting Robot (2x)

MagNA Pure LC DNA isolator

Roche

Real time PCR
ABI Prism SDS 7000

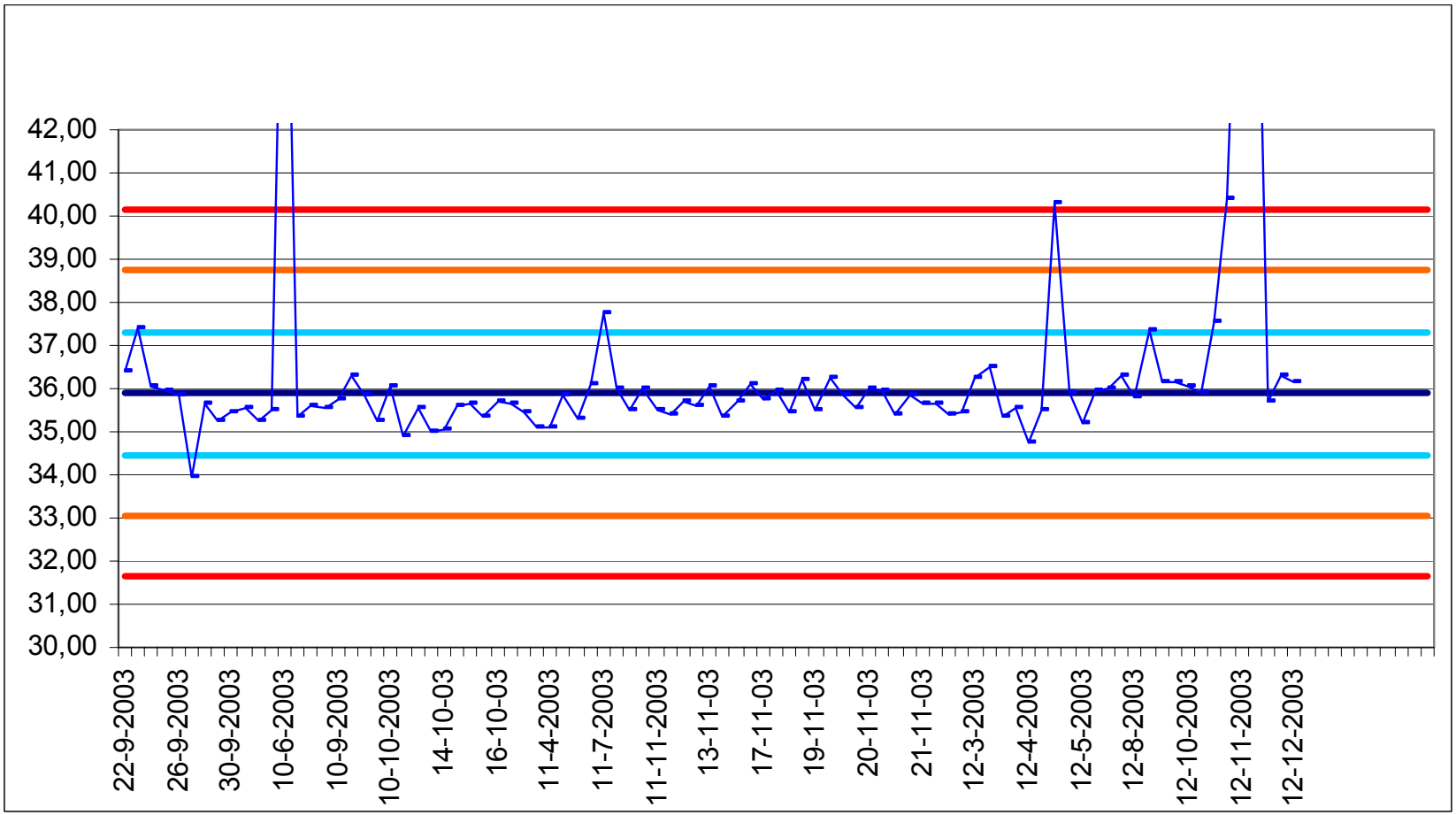
Technical details

- Anti-coagulated blood samples (sent by post or courier at RT)
- Centrifuged at 2840 rpm for 10 min without brake
- Centrifuged at 4000 rpm for 20 min
- DNA isolated from 1 mL plasma
- DNA eluted in 55 µL
- 15 µL in *RHD*-exon 7 PCR (50 µL) in **triplicate**

Controls

- 2 “runcontrols” (plasmapool derived from pregnant D-neg women with D+ fetuses) ; 1:2 and 1:4 diluted
- Internal Positive Control (IPC) in RQ-PCR to test for PCR-inhibitors

Ct-values of 1:2 runcontrol (Shewartcard) (86 MagNA Pure runs)



Capacity

- In a regular working day:
  - 4 MagNA Pure runs = 4x30 patientsamples
  - => 31.200 / year
- Hands-on time of technician: 2,5 hrs / day
  - 1x 40 minutes for centrifugation step / Tecan robot
  - 4x 5 minutes for starting MagNA Pure
  - 4x 3 minutes for starting pipetting into Taqmanplate
  - 4x 5 minutes for starting Taqman run
  - 4x 15 minutes for analyzing data

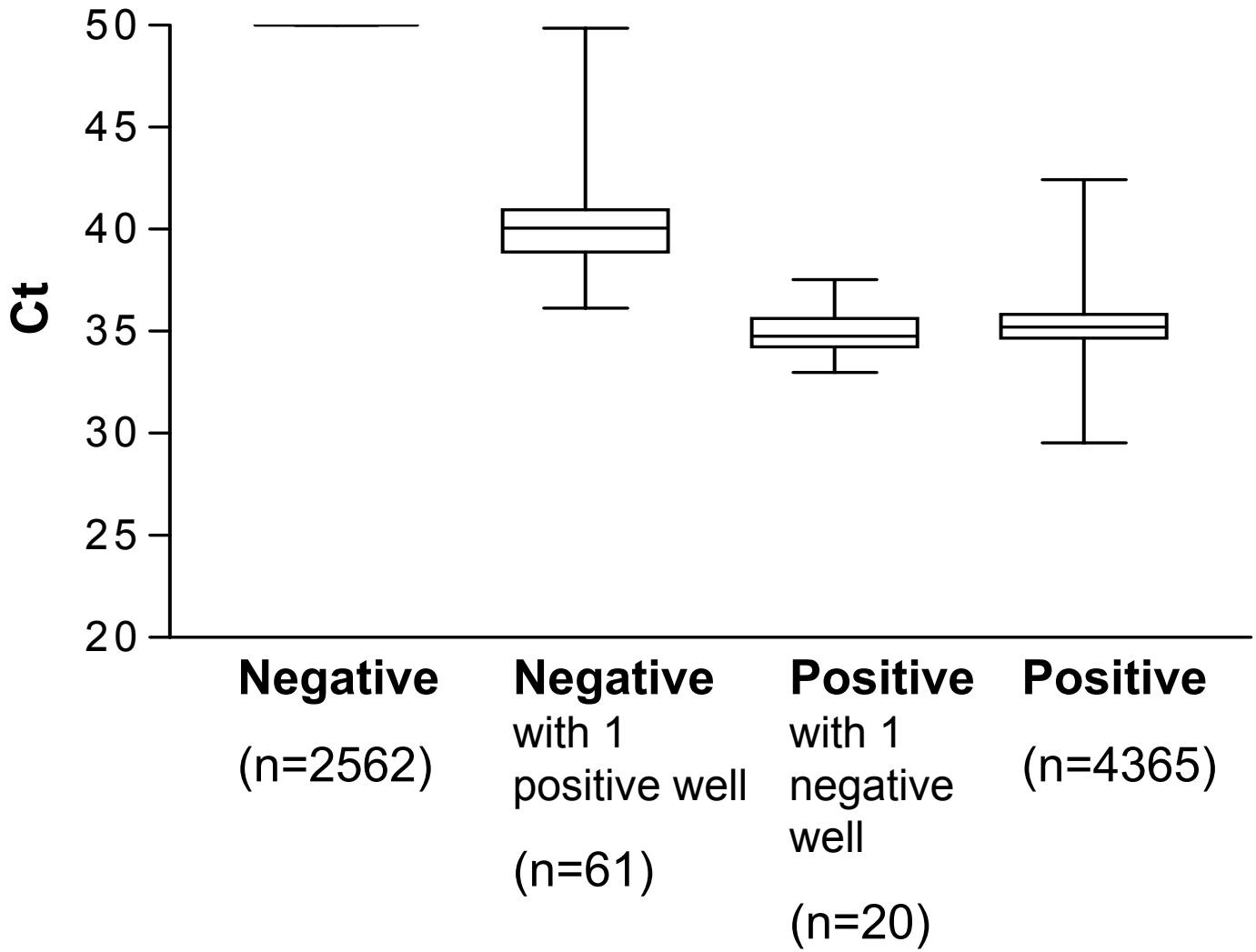
Study-Design

- >2500 D-negative pregnant women, whose blood was sent to CLB for 28-30th week-antibody screening
- Plasma was tested in *RHD*-PCR and all (serologically confirmed) D-neg women (without IEA) were sent questionnaires on cord blood serology to be completed after delivery
- Further testing of discrepant results (review of serology, buccal swabs of newborn)

Results

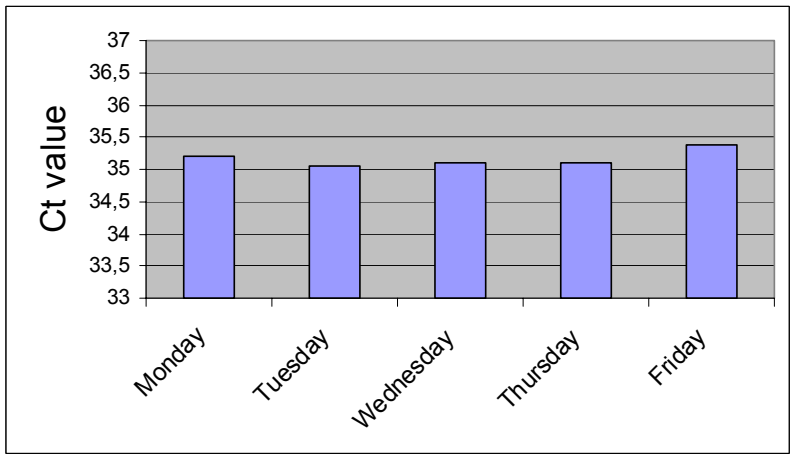
- Plasma of 2415 supposed D-neg women has been tested
  - 35 women (**1.44** %) were D-positive according to serology at CLB
    - 10 weak D (n=7) or variant D (n=3)
    - 25 Normal D
  - In the 2380 D-neg women the fetus was typed as
    - D-positive in PCR :1465
    - D-negative in PCR: 915

Clear discrimination between positive and negative PCR results

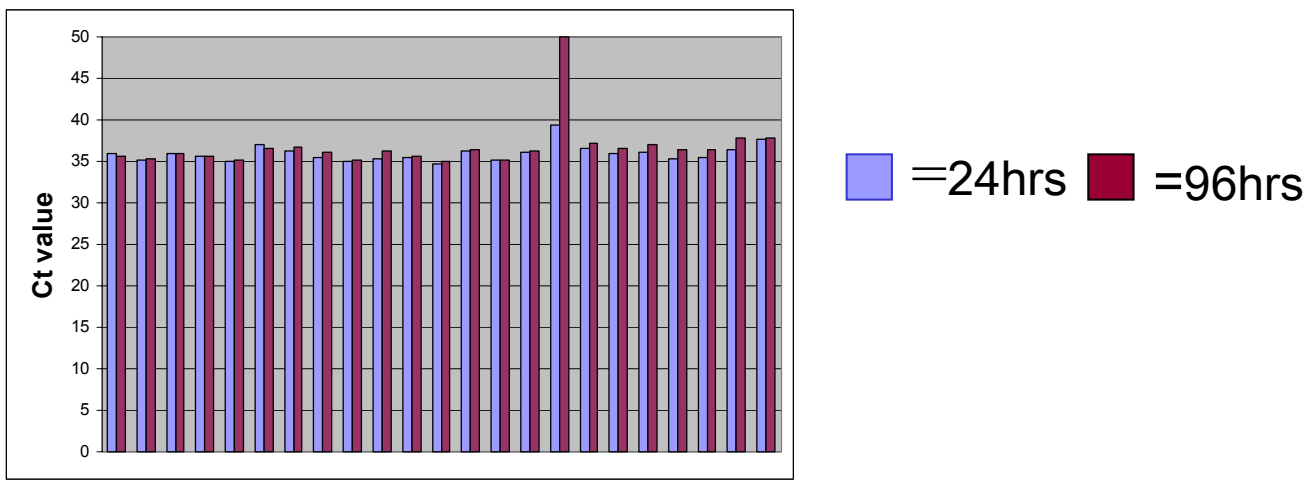


Effect of shipping / storage of blood

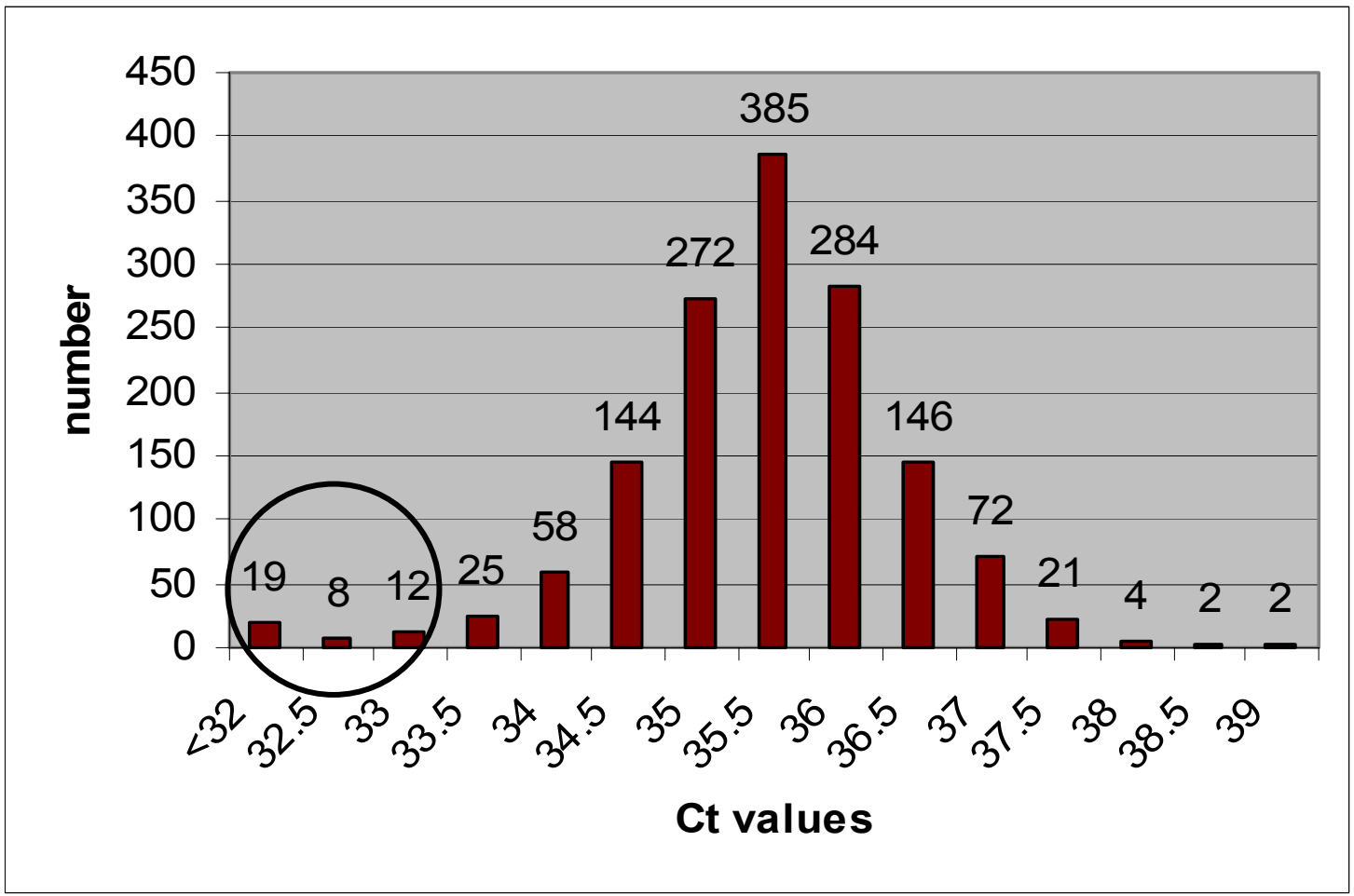
- Mean amount of fetal DNA is comparable for all samples sent in at different days



- Effect of storage of whole blood at RT (n=22)



Median fetal DNA plasma concentration in **30**th wk =**400 pg/mL** range 40-2800 pg/mL , **n=1443**



39 samples with fetal DNA concentration > 1500 pg/mL

Is this fetal DNA or is the mother carrying a D-negative *RHD*-gene

⇒DNA isolated from maternal leukocytes and tested for variant *RHD* genes

- 20 women 1500-3000 pg:
  - all RhD-negative
- 19 women > 3000 pg /mL
  - 4 women no *RHD* gene -> increased level of fetal DNA (0.28%)
  - 15 women carried an *RHD* gene (0.63%)

15 out of 2380 serologically D-negative women carried an *RHD* gene

Molecular basis:

6 x *RHD* pseudo gene

1 x RHD_{el} (IVS3+1G>A, splice site mutation)

3 x *RHD* type VI type 2

1 x *RHD* type VI type 1

1 x *RHD* type VI type 4

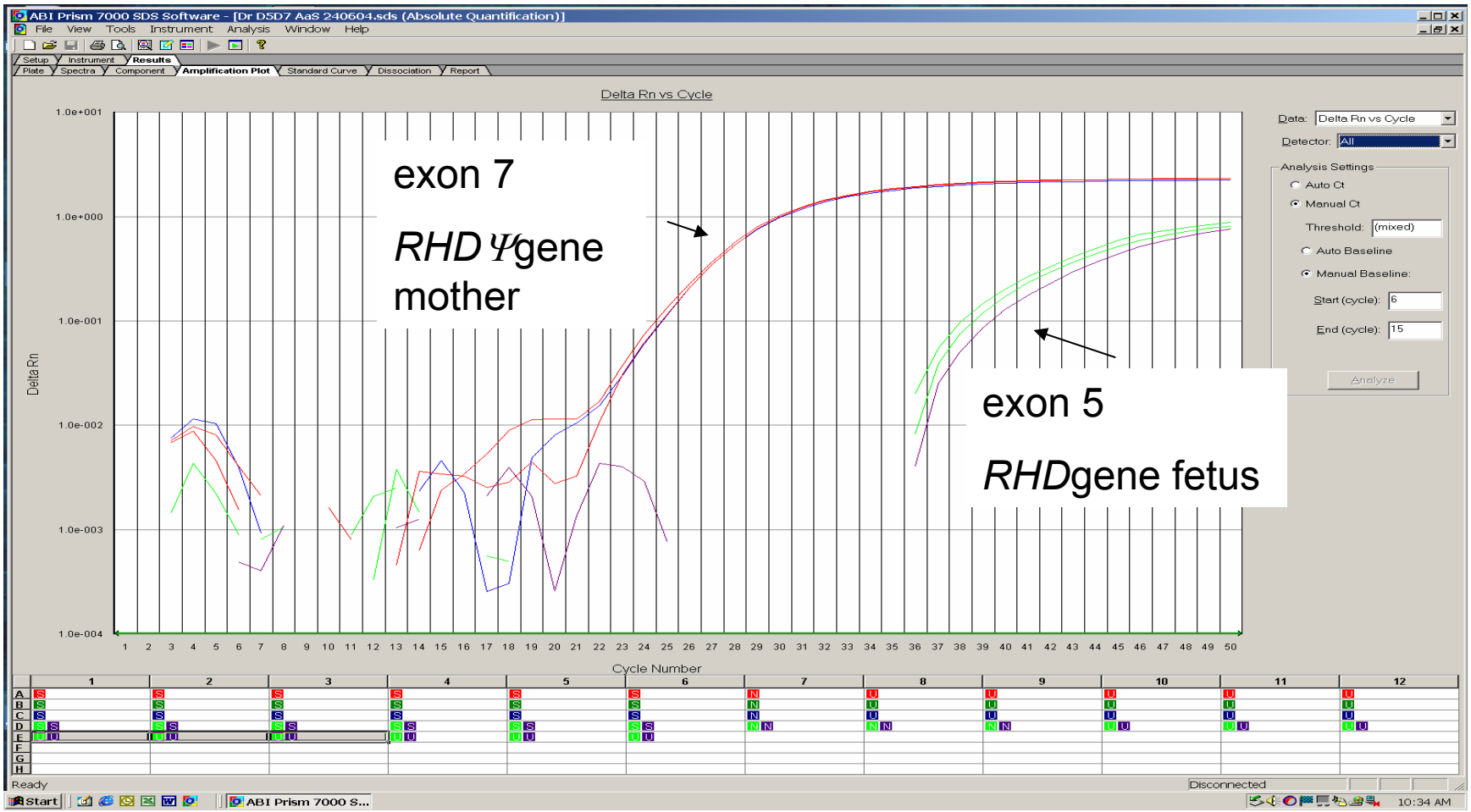
1 x weak D type 1

1 x weak D type 11

1 x weak D type 17

0.34%
1:300

RHD-gene positive fetus in *RHD Ψ*-gene positive D-negative mother can be recognized by exon5 – exon 7 PCR *(Finning et al. Transfusion2001)*



Comparison of PCR results with Cord blood serology

- Questionnaire with two questions
  - What is the RhD factor of the newborn in cord blood?
  - Have you received anti-D within 48 hours after delivery?

- 1297 / 2359 (55%) women responded (April 08, 2004)

- Originally
  - 31 questionnaires were not completed / inconsistent
  - 21 discrepant results between PCR and serology
  - 1245 concordant result

Examination of discrepant results

- Serology D- and PCR D+ (n=10)
  - 5 incorrect questionnaire
  - 5 serological D-neg (0.38 %) => buccal swab

- Serology D+ and PCR D- (n=11)
  - 4 incorrect questionnaire
  - 4 serology is not reliable
  - 3 serological D-pos

} buccal swab will be tested

Concordant results in 99.1% of the tested samples (n=1257)

	Cord blood D+	Cord blood D-
PCR D+	787	7
PCR D-	5	458

Conclusions

- High throughput non-invasive fetal RhD genotyping in 30th week is at least as reliable as cord blood serology (>99% diagnostic accuracy)

- Assay costs for reagents and equipment are below 15 euro / assay

  ➔ This assay can be used to restrict antenatal prophylaxis to D-neg women pregnant of a D-pos child

  ➔ Postnatal cord blood typing can be omitted, at least in all women with a D+ PCR result . Postnatal prophylaxis can then be given directly after delivery, which might increase its effectiveness

Acknowledgements

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NWO

Biological validation of automatic fetal RhD typing in 16-20th week (n=192)

Amniotic fluid or cord blood serology

Plasma PCR	RHD +	RHD -
RHD +	123	0
RHD -	0	69