Aus dem Institut für Transfusionsmedizin
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

High-throughput Testing for the Determination of the Fetal Rh Factor in Maternal Plasma

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 21.11.2008

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Chapter 1   **Introduction**

1 .1 Molecular background of Rh blood group system

The Rh blood group system is the most complex blood group known. Rh antigens are encoded by a pair of highly homologous genes, *RHCE* and *RHD*, which are located at chromosome 1p34.3-36.1 (Cherif-Zahar B, et al.1991). The *RHCE* gene gives rise to the C/c and E/e polymorphism, while the *RHD* gene encodes the D polypeptide. Each gene consists of 10 exons, and there are only 43 nucleotide differences between them. The *RHD* gene and *RHCE* gene face each other by their 3' end, and are separated by about 30 kb pairs that contain the SMP1 gene. Furthermore, the *RHD* gene is flanked by so called *Rhesus boxes*, with a length of approximately 9 kb pairs.

In most cases, both *RHD* and *RHCE* genes are present at the *RH* locus in RhD-positive individuals, whereas in RhD-negative individuals the *RHD* gene is deleted. In RhD variants, a *RHD* gene is also present, although mostly point mutations, deletions or hybrid alleles are found (Legler TJ et al. 1998; Flegel WA 2006, Wagner FF, 2000). However, genuinely D-negative individuals do not always lack the *RHD* gene. Portions of *RHD* or the entire *RHD* gene with or without point mutations have been found.

The deletion of *RHD* is the most common reason for the D-negative phenotype in the Caucasian population. However, about 67% of D-negative black Africans have a complete, but inactive *RHD*, the *RHD*-pseudogene *RHDψ* (Daniels G 2004). PCR assays reported to amplify *RHD* on exon 7 or the 3' non-coding region (NCR) of *RHD* will falsely classify fetuses with *RHDψ* as D-positive. In the initial genotyping studies, *RHDψ* was not analyzed. Finning et al. and Legler et al. published the detection of both *RHD* and *RHDψ* with real-time PCR in 2002.

1.2 Blood exchange between mother and fetus during pregnancy
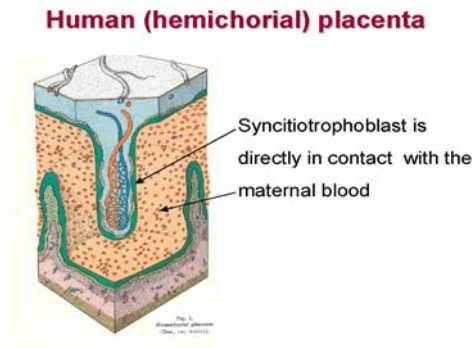
Fig.1-1 Blood exchange between mother and fetus in pregnancy

The exchange of nutrition and oxygen between mother and fetus occurs through the placental barrier during pregnancy [Fig.1-1]. Although the placental barrier separates maternal and fetal cells, a few fetal cells can cross the placental barrier and enter into the maternal circulation. In 1893, Schmorl first documented the presence of fetal-derived trophoblast cells in the lungs of women who died from pre-eclampsia. It is also now known that a pregnant woman is exposed to large amounts of fetal DNA during pregnancy (Lo YMD 1997; Lagona F et al. 1998; Holzgreve W et al. 1992, Bianchi and Lo, 2001).

1.3 Hemolytic disease of the fetus and newborn

A D-negative pregnant woman carrying a D-positive child may develop anti-D antibodies. When her next child is D-positive, it may be affected by the anti-D antibodies, which will cross the placenta. The child will suffer from the risk of many diseases, such as haemolytic anemia, low oxygen, liver failure, low serum protein and edema (Randen I. et al, 2003). If the child is not treated with intrauterine red blood cell transfusion, it will probably suffer from lethal diseases. Today the mortality of an affected D-positive child is about 2-3%.

Prior to the 1970s, haemolytic disease of the fetus and newborn (HDFN) was a significant cause of fetal and neonatal morbidity and mortality. The incidence of HDFN is now about one in 21,000 births in Europe. In England and Wales, about 500 fetuses develop HDFN each year, of

which 25-30 babies die, and at least 20 pregnancies per year are lost to spontaneous abortion before 24 weeks of gestation. When a D-negative woman with anti-D is pregnant, it is beneficial to know the D phenotype of her fetus in order to initiate frequent monitoring of D-positive fetuses and apply intrauterine transfusion if required (Daniels G et al. 2004, Lo YMD 1997).

1.4 Prevention of haemolytic disease of the fetus and newborn
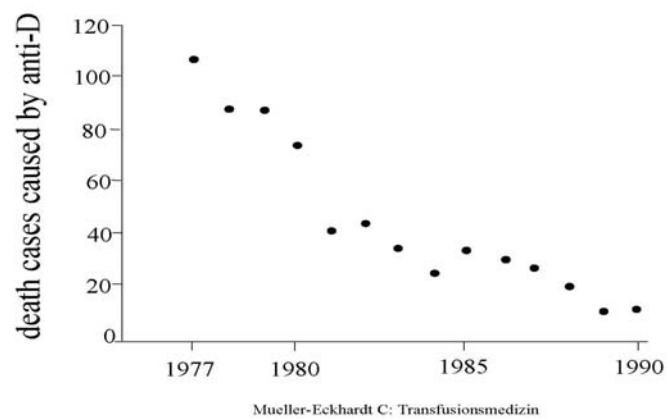
Fig.1-2 Success of anti-D prophylaxis

To prevent primary anti-D immunization in D-negative women during pregnancy, it is common practice to offer routine antenatal anti-D prophylaxis (RAADP), usually at 28-34 weeks of gestation, and within 72h after delivery in case of a D-positive newborn. With the development of the anti-D prophylaxis and due to improved treatment strategies including intrauterine red cell transfusion fewer deaths occur due to anti-D alloimmunization (Fig.1-2).

If no anti-D prophylaxis is given, anti-D immunization occurs in about 13.2% per D-positive pregnancy. If only post-delivery anti-D prophylaxis is given, the immunization rate decreases to about 1.8%. In women who receive both antenatal and postnatal RhD prophylaxis, the immunization rate is about 0.14%(Bowman J, 2003). Although the implementation of routine anti-D prophylaxis was a benefit for many fetuses in the past, this blood product is frequently not indicated. In a predominantly Caucasian population, 15-18 % of all pregnancies occur in D-negative women (Mackenzie IZ et al. 1999). In 40% of all D-negative cases anti-D prophylaxis is not indicated because the fetus is D-negative. Because the phenotype of the fetus is not known, about 40% of women carrying a D-negative fetus will receive this therapy unnecessarily.

Consequently, it would be beneficial if a method (especially a high-throughput method) was available for determining the fetal D type in all pregnant D-negative women (Costa JM et al. 2002; Daniels G, 2004; Daniels G et al. 2004; Gautier E et al. 2005; Faas BH et al. 1998; Finning KM et al. 2002; Hromadnikova I et al. 2005; Birch L et al, 2005; Pertl B et al, 2000; Singleton BK et al, 2000; Van der Schoot CE et al. 2004; van der Schoot CE et al. 2006; Zhong XY et al. 2001; Lo YMD et al. 1998). Meanwhile, in the case of a D+ fetus the prenatal determination of the fetal D-status would allow immediate anti-D prophylaxis after birth instead of waiting for a blood group determination from cord blood.

1.5 Antenatal genotyping of the fetal RhD type

As there is an exchange of nucleated cells and cell-free DNA between the mother and fetus during pregnancy, fetal cells in maternal blood and fetal DNA in maternal plasma offer a non-invasive source of fetal material for prenatal diagnosis. Maternal plasma testing could eventually enable the screening of all D-negative pregnant women, thereby confining the administration of prophylactic anti-D only to those pregnancies in which it is needed (Gautier E et al. 2005; Finning KM et al. 2002; Finning KM et al. 2004).

Determining fetal D type from fetal DNA in maternal plasma in all pregnant D-negative women would be valuable for several reasons (Daniels G et al. 2004): Firstly, the availability of prophylactic anti-D is at present insufficient to meet the increase in demand. The increase in demand results from the decision in several countries to add antenatal Rh-prophylaxis to the current postnatal prophylaxis. Secondly, it is unclear as to whether blood plasma based products could be contaminated with Variant Creutzfeldt-Jakob disease (vCJD) and indeed the UK National Blood Service has ceased using plasma isolated from British blood donors, and imports its source plasma from overseas as a precautionary measure. Thirdly, in alloimmunized pregnancies the risk of transplacental haemorrhage associated with diagnostic invasive procedures such as amniocentesis can be avoided.

1.6 How to determine the RhD type of the fetus

The procedures for determining the RhD type of the fetus can be classified into 2 categories. The first one is an invasive procedure, such as amniocentesis or chorionic villi sampling. In this procedure, a small sample of amniotic fluid is drawn out of the uterus through a needle. The fluid is then analyzed to detect genetic polymorphisms in the fetus. However, the procedures for obtaining these materials are expensive and invasive. For example, amniocentesis is associated with a 0.5-1% risk of spontaneous abortion and a 17% risk of transplacental haemorrhage. Chorionic villus sampling is associated with a higher risk of spontaneous abortion, but a reduced risk of transplacental haemorrhage.

A different procedure is the use of non-invasive methods. The non-invasive methods can also be divided into two categories, the cell-based method and cell-free method.

The "modern era" of fetal cell isolation from maternal blood began in 1969 when Walknowska detected lymphocytes carrying an X and a Y chromosome in the peripheral blood of pregnant women (Walknowska, et al.1969). During pregnancy there is a two-way exchange of cells between the fetal and maternal circulation. Therefore, fetal cells may be obtained from maternal peripheral blood for genetic analysis, without endangering the fetus. However, fetal cells in maternal blood are extremely rare and technically difficult to separate from their maternal counterparts. This approach has not proved sufficiently reliable for routine clinical applications (Daniels G et al. 2002).

Lo YMD et al. (1997) have shown that in addition to the presence of fetal cells in maternal blood, cell free DNA is also present in maternal circulation. Using a quantitative PCR assay, fetal DNA has been demonstrated to be present in high concentration in maternal plasma. Because of the relative abundance of this fetal genetic material, which is present in concentrations several orders of fetal magnitude higher than those fetal cells, which are being exchanged, the determination of fetal genetic loci that are totally absent from the maternal genome is relatively easy (Li Y et al. 2004). This observation suggests that cell-free fetal DNA analysis can be applied clinically for the non-invasive prenatal diagnosis of certain disorders, including haemolytic disease resulting from Rh blood group incompatibility. (Legler TJ et al. 2002; Lo YMD et al. 1999). The international blood group reference laboratory in Bristol, UK has introduced a fetal blood group genotyping service for immunized pregnant women with

heterozygous partners in 2002 (Finning KM et al. 2002). Similar services are available in Amsterdam for The Netherlands and Prague in Czechia. However, it had not been implemented in Germany at the beginning of this study.

The fetal DNA can be extracted from maternal plasma or serum. A potential important difference between different research groups concerns the choice of plasma or serum for analysis. It has been shown that the absolute concentration of fetal DNA in maternal plasma and serum is comparable. However, greater amounts of maternal DNA have been found in maternal serum than plasma. These results indicate that a higher maternal background is present in serum. This may be detrimental for the detection of fetal DNA, especially when less sensitive detection methods are used (Lo YMD. et al. 1999).

1.7 The method of genotyping fetal RhD by the detection of fetal DNA

1.7.1 The physiology of fetal DNA in maternal plasma

Fetal DNA in maternal plasma

The placenta can be regarded as a pseudomalignant tissue. Lo YMD et al. (1997) demonstrated that placental derived fetal DNA is present in maternal plasma. This group has found that the prediction of the fetal *RHD* status is reliable from the second trimester onwards. The earliest detection of fetal DNA in pregnancy was reported in 2003 after 5 weeks of gestation (Rijnders RJ et al. 2003).

The fetal-maternal DNA ratio in plasma increases as the gestational time increases (Lo YMD. et al. 1998). At 11-17 weeks, the mean percentage of fetal DNA of total DNA (including fetal and maternal DNA) in the maternal plasma is 3.4 % (range: 0.39 – 11.9%), and in 37-43 weeks the rate is 6.2 % (range: 2.33% - 11.4%). The concentration of fetal DNA in the maternal plasma increases similarly during pregnancy. In the 16th week the mean concentration is 149 pg/ml (range 23-952, i.e. 23 geq/ml, n = 120), and in the 30th week the mean concentration is 522 pg/ml (range 20-4640, i.e. 79 geq/ml, n = 299).

From these data it has become clear that in some cases the concentration of fetal DNA in maternal plasma is at the analytical limit of detection. Therefore, single testing of fetal genetic

markers from circulating DNA, RNA or cells is not absolutely safe. As a consequence most laboratories perform multiple analyses (3-12 replicates) in order to improve their diagnostic sensitivity.

Rapid clearance of fetal DNA from maternal plasma
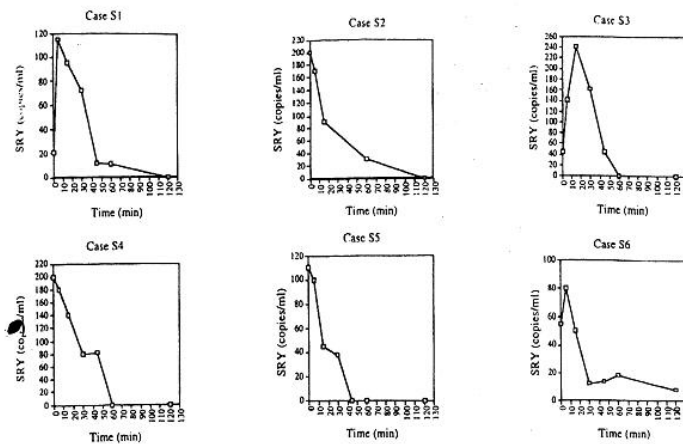
Figures1-3, clearance of fetal DNA from maternal plasma
T1/2 = 16 minutes (range 4-30)

When fetal DNA is extracted from the maternal plasma and used to determine the RhD status, a question might arise. Could there be a false positive result due to the remainder of DNA from previous pregnancies? Lo YMD et al. (1999) demonstrated that the fetal DNA is cleared rapidly from maternal plasma after delivery. Figures1-3 show the amount of fetal DNA in maternal plasma at different time points after delivery. The results clearly show that there is no fetal DNA in maternal plasma after 30 minutes (T1/2 = 16 minutes).

The rapid clearance of cell-free fetal DNA from maternal blood suggests that it is extremely unlikely that fetal DNA persists from one pregnancy to the next, unlike certain fetal nucleated cell populations which have been demonstrated to persist in the mother for years following delivery (Lo YMD. et al. 1999).

The size distribution of fetal and maternal DNA fragments in maternal plasma
Plasma DNA molecules are mainly short DNA fragments. The DNA fragments in the plasma of pregnant women are significantly longer than those in the plasma of non-pregnant women, and the maternal-derived DNA molecules are longer than the fetal-derived ones. Li Y et al. investigated the size distribution of fetal and maternal DNA fragments in maternal plasma using

real-time PCR (Li Y et al. 2004). The median percentages of plasma DNA with size >201bp were 57% and 14% for pregnant and non-pregnant women, respectively. The median percentages of fetal-derived DNA with sizes >193bp and 313bp were 20% and 0% respectively in maternal plasma. Li Y et al. also observed that the circulatory DNA has apoptotic characteristics, displaying a typical ladder obtained by nucleosomal cleavage. They also observed that circulatory fetal DNA is generally of a smaller size than maternally derived cell-free DNA fragments. By exploiting this observation, they have shown that even a simple strategy, such as size separation using conventional agarose gel electrophoresis and subsequent PCR analysis, can lead to the selective enrichment of circulatory fetal DNA sequence. This in turn can be used for the determination of DNA polymorphisms that are masked by maternal sequence in the native plasma samples. Their data indicated that selective enrichment of circulatory fetal DNA sequences may be possible by examination of DNA fragments with a size less than ~300 bp.

Analysis of size-fractionated DNA (≤0.3 kb) from maternal plasma facilitated the detection of paternally and maternally inherited micro-satellite markers. The different size distributions of DNA in the plasma of pregnant women may open up a possible way to enrich fetal DNA by size fractionation of DNA extracted from the plasma of pregnant women. Such enrichment could also allow the prenatal diagnosis of autosomal recessive disorders and chromosomal aneuploidies (Chan KCA et al. 2004).

1.7.2 Methods for the detection of fetal DNA from maternal plasma

The principle of the extraction of fetal DNA from maternal plasma by magnetic particles
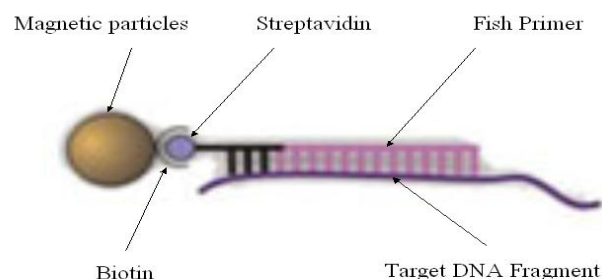
Fig.1-4 The principle of the isolation of DNA by the specific fish primer

Since the double-strand DNA will become single-strand at a high temperature (90˚C) and annealed at about 60˚C, according to the principle of base-pair (bp) matching, oligonucleotides can be designed, which can match the target DNA fragments, to anneal with the target DNA fragment specifically. Furthermore, these oligonucleotides can be coupled with biotin. Thus biotinylated DNA complexes can be bound to the surface of the magnetic particles coated with streptavidin. When a magnetic field is applied to the magnetic particles, the target DNA can be collected by controlled movement of the magnetic particles. [Fig.1-4]

High-throughput nucleic acid amplification techniques (NATs) are required for the detection of viral genomes in individual blood donations and might be helpful in any virological laboratory. In a pilot study, Legler TJ et al. (1999) demonstrated that high-throughput automated HCV-RT-PCR is practicable for testing individual blood donations. Additionally, the PCR approach described could easily be adapted to the detection of other viral genomes by the use of specific primers.

The challenge and problem of this method
A caveat of current investigations is that the overwhelming amount of circulatory DNA in the maternal circulation is of maternal origin (>90%), which has rendered the differentiation of more subtle genetic differences between mother and child considerably more difficult (Li Y et al., 2004). In order to increase the sensitivity and accuracy, the fetal DNA should be extracted from larger volumes of maternal plasma.

1.8 Objective

In this thesis, DNA extraction methods for the isolation of free fetal DNA of D-positive fetuses from the maternal plasma of D-negative pregnant women will be evaluated and optimized. Ideally the methods should allow automated high-throughput testing. This would be cost effective, as it would save wastage of anti-D immunoglobulin, a valuable and expensive resource. It would also be beneficial to the D-negative patients, who would avoid unnecessary therapy with blood products.

Objectives include:
1. Optimization of nucleic acid extraction and enrichment of fetal DNA from maternal plasma

2. Optimization of the methods towards high-throughput procedures

3. Improving the sensitivity, feasibility and reproducibility of the method

4. Decreasing the cost and time required for fetal *RHD* detection

5. Automation of the method, which should be suitable for routine clinical applications.

1.8.1 Optimization of the QIAamp DSP Virus Kit for the extraction of fetal DNA from maternal plasma

The CE marked QIAamp DSP Virus Kit protocol (QIAGEN, Hilden, Germany) has been developed for viral RNA and DNA isolation from plasma. It is useful for extracting nucleic acids from 500µl plasma. This protocol is a sensitive and reproducible method for viral RNA isolation. In order to increase the sensitivity and reproducibility of prenatal diagnosis, the QIAamp DSP virus kit was evaluated and optimised for the isolation of fetal DNA from maternal plasma.

1.8.2 Fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

The method of non-invasive prenatal diagnosis in maternal plasma has faced the challenge of the overwhelming presence of maternal DNA sequences in the circulatory DNA (Li Y et al. 2004). It is very important to separate the fetal DNA from the total DNA that is extracted from maternal plasma in order to overcome this challenge. However, so far, to our knowledge, most of the current methods used cannot separate fetal DNA. Here, a method (Hybridisation Capture and Magnetic particles) was explored to enrich fetal DNA separated from the circulatory DNA of maternal plasma according to different size distributions of fetal and maternal DNA and specific fish primers.

In this part of the work, a specific fish primer was designed to capture the fetal DNA. The fetal DNA was isolated with the target specific capture oligonucleotides and magnetic particles, which are sensitive to short DNA fragments by optimisations. Fetal and maternal DNA was quantified testing *RHD* specific polymorphisms in exon 7 with real time PCR. At least 8 replicates were

analysed using SPSS software. The amount of fetal and maternal DNA in maternal plasma was calculated from a curve using human DNA as a standard. This technology was first established manually and subsequently automated using a commercially available liquid handling robot.

1.8.3 Evaluation of new fetal DNA extraction methods in an international survey

An international survey was organized, funded by the 6th framework program, so that the new fetal DNA extraction methods could be evaluated. Participants of this survey were mainly partners of the Network of Excellence SAFE(Special Non-Invasive Advances in Fetal and Neonatal Evaluation). SAFE is funded with the key aim of implementing routine Non-Invasive Prenatal Diagnosis (NIPD) and cost effective neonatal screening throughout the EU and beyond. Companies, which produce robotic instrumentation for the extraction of DNA, were invited to participate in this survey.

Chapter 2   **Materials and Methods**

2.1. Materials

2.1.1. Maternal blood samples

Diagnostic blood samples were collected in EDTA tubes from D-negative pregnant women and sent to the Wagner Stibbe and Partner laboratory, Göttingen, Germany. Before centrifugation the samples were stored at 2-8℃. Plasma was separated from cells and after diagnostic tests were completed, 1-2 ml was stored in deep-well microtiter plates for testing later in pregnancy at -20°C. Due to the policy of this laboratory this residual plasma is discarded after 3 months. For this study those microplates were used in combination with the results from the serological maternal blood group result (anonymized computer lists) including the maternal D-status. The study protocol was approved by the local ethical board (no. 26/9/04). Within 2 weeks, 1000 samples were collected from D-negative women. 30 plasma-pools were prepared after thawing the plates. Plasma pools were centrifuged at 2840 rpm (1600g) for 10 mins without brake. The plasma fractions were removed and centrifuged again at 3600 rpm (2800g) for another 20 mins. Finally, several 2 ml aliquots were pipetted from each plasma-pool.

Each plasma pool was analyzed by the QIAamp DSP Virus Kit (Qiagen) and D-specific real-time PCR. When the Ct (=cycle threshold) value of the fetal DNA in the plasma pool was evaluated as being in the range between 30 and 40, the plasma sample would be used in the experiments. If the Ct value was lower than 30, the plasma pool would be discarded, as it could not be excluded that the pool was contaminated with *RHD* positive plasma.

2.1.2 *RHD* negative control plasma

Citrate plasma was pooled from 30 male D-negative plasma donors with blood group AB. Plasma pools were centrifuged at 2840 rpm for 10 mins without brake. The plasma fraction was removed and centrifuged again at 3600 rpm for a further 20 mins.

2.1.3 Genomic DNA standard

The samples of *RHD* positive DNA used here with defined concentration were purchased from

Roche (Basel, Switzerland). The concentration of Human Genomic DNA is 0.2mg/µl in 10mM Tris-HCl, 1mM EDTA. pH=8.0. Dilutions of this reference material were used for standard curves and quantitative analysis. A conversion factor of 6.6 was used when pg values were transformed to genome equivalents (geq).

2.1.4 PCR mixture

The *RHD* specific exon7 real-time PCR was performed as previously published (Legler TJ et al. 2002). In brief 50µl reactions consisted of 1.5 U Taq polymerase (TaqMan PCR core kit, Applied Biosystems, Foster City CA), 200µM dNTPs, buffer, 3.0 mM $MgCl_2$ primers and probe (Table 2-1), 2µL (HCMP method) and 15µL (Qiagen method) template, respectively.

Table 2-1: Amplification Primers and Probe for *RHD* real-time PCR

Primer designation	Amplification Primer sequence	Label	*RHD* region
D7b-*sense*[1]	CTCCATCATGGGCTACAA	none	exon 7
D7b-*antisense*[1]	CCGGCTCCGACGGTATC	none	exon 7
D7b-*probe*[1]	AGCAGCACAATGTAGATGATCTCTCCA	FAM-TAMRA	exon 7

[1]Legler et al. 2002

2.2. Methods

2.2.1 Extraction of cell-free DNA from plasma using the QIAamp DSP Virus Kit

Nucleic acid extraction was performed mainly according to the recommendations of the manufacturer [Fig.2-1]. The amount of carrier RNA, the type of elution buffer and amount of protease was modified in separate experiments.

    Step 1. Pipet 100 µl QIAGEN Protease (QP) into a Lysis Tube (LT).

    Step 2. Add 500 µl plasma samples to the Lysis Tube.

    Step 3. Add 500 µl Lysis Buffer (AL) to the Lysis Tube, close the lid, and mix by pulse-vortexing for 15 s.

    Step 4. Incubate at 56°C (±1°C) for 20 min.

Step 5. Centrifuge the Lysis Tube (LT) for ≥5 s at full speed to remove drops from the inside of the lid.

Step 6. Change gloves and open the Lysis Tube (LT) carefully.

Step 7. Add 600μl ethanol(96–100%) to the Lysis Tube(LT), close the lid, and mix thoroughly by pulse-vortexing for ≥15 s. Incubate for 5 mins (±1 min) at room temperature (15–25°C).

Step 8. Centrifuge the Lysis Tube (LT) for ≥5 s at full speed to remove drops from the inside of the lid.

Step 9. Insert the QIAamp MinElute Column into the VacConnector (VC) on the vacuum system (see Fig.2-2). Insert a Column Extender (EXT) into the open QIAamp MinElute Column.

Step 10. Change gloves and open only one tube at a time.

Step 11. Carefully apply the entire lysate from step 7 into the Column Extender (EXT) of the QIAamp MinElute Column without wetting the rim. Avoid touching the QIAamp MinElute Column membrane with the pipet tip.

Step 12. Switch on the vacuum pump. After the lysate has been drawn through the QIAamp MinElute Column, open the valve of the vacuum system, and release the vacuum.

Step 13. Apply 600 μl Wash Buffer 1 (AW1) to the QIAamp MinElute Column. Carefully remove and discard the Column Extender (EXT), and close the valve of the vacuum system. After Wash Buffer 1 (AW1) has been drawn through the QIAamp MinElute Column, open the valve, and release the vacuum.

Step 14. Apply 750 μl Wash Buffer 2 (AW2) to the QIAamp MinElute Column without wetting the rim. Avoid touching the QIAamp MinElute Column membrane with the pipet tip. Leave the lid of the column open, and close the valve of the vacuum system. After Wash Buffer2 (AW2) has been drawn through the QIAamp MinElute Column, open the valve, and release the vacuum.

Step 15. Apply 750 μl ethanol (96–100%) to the QIAamp MinElute Column without wetting the rim. Avoid touching the QIAamp MinElute Column membrane with the pipet tip. Leave the lid of the column open, and close the valve of the vacuum system. After ethanol has been drawn through the QIAamp MinElute Column, open the valve, and release the vacuum.

Step 16. Close the lid of the QIAamp MinElute Column, remove it from the vacuum system, and discard the VacConnector (VC). Place the QIAamp MinElute Column in the Wash Tube (WT) saved from step 9, and centrifuge at full speed (approximately 20,000 x g, or 14,000 rpm) for 1 min to dry the membrane completely. Discard the Wash Tube (WT) containing the filtrate.

Step 17. Place the QIAamp MinElute Column in a new Wash Tube (WT), and incubate with the lid open at 56°C for 3 min to evaporate any remaining liquid.

Step 18. Place the QIAamp MinElute Column in a clean Elution Tube (ET), and discard the Wash Tube (WT). Carefully open the lid of the QIAamp MinElute Column, and apply 20 µl distilled water to the center of the membrane. Close the lid and incubate at room temperature (15–25°C) for ≥3 mins. Centrifuge at full speed (approximately 20,000 x g, or 14,000 rpm) for 1 min to elute the viral nucleic acids.
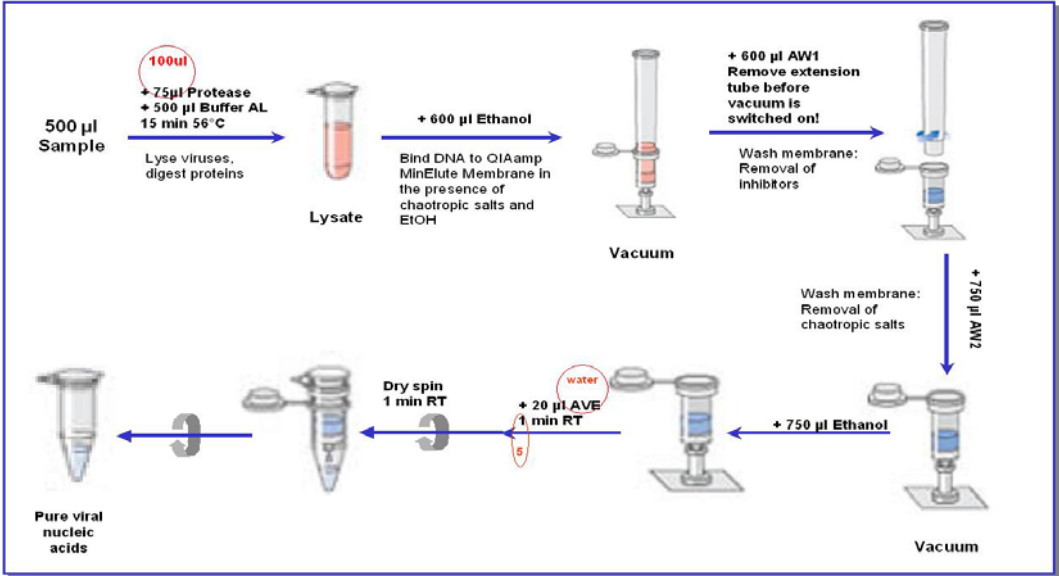
Fig.2-1 The procedure of QIAamp DSP Virus Kit (The procedures marked in red are those modified in this study)

Fig.2-2 The QIAvac 24 Plus, Connecting System, and Vacuum Pump allows collection of fetal DNA and RNA

2.2.2 Optimization of the QIAamp DSP Virus Kit protocol

In initial experiments, it was found that it was difficult to draw the lysate through the membrane in the QIAamp MinElute Column. It was suspected that the protein was not completely denatured and caused clogging of the pores. Therefore, the amount of protease was increased from 75 µl to 100 µl.

In order to evaluate the influence of the amount of protease, 24 samples were assessed. One aliquot of each sample was tested with 75 µl protease; another aliquot of each sample was tested with 100 µl protease. The experimental details were the same as in Steps 1-14 of Chapter 2.1 of Materials and Methods. In step 12, the time required for the lysate to pass through the membrane was recorded for each sample. Furthermore, the Ct values in real-time PCR were compared.

2.2.3 Influence of Carrier RNA

The carrier RNA was prepared according to the handbook of QIAamp DSP Virus Kit (April 2004). In this experiment, it was studied how carrier RNA influences the isolation of fetal DNA. Eight pooled samples were tested. One aliquot of each sample with carrier RNA was tested; another aliquot of each sample without carrier RNA was tested. The experiment details were shown in Chapter 2.1 of Materials and Methods. According to the protocol carrier RNA is added in Step 3, alternatively, this step is omitted.

2.2.4 Comparison between the DNA elution in water and in AVE buffer

In this experiment, the influence of the DNA elution solution was studied. 14-pooled samples were tested. One aliquot of each sample was tested when the DNA was eluted in water (step 18, Chapter 2.1 of Materials and Methods); another aliquot of each sample was tested when DNA was eluted in AVE buffer.

2.3 Fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

2.3.1 Materials

Extraction buffer

The extraction buffer used was composed of 6M Guanidine Hydrochloride (pH was adjusted to 9.2 by NaOH solution, except that in the experiment of studying the influence of the pH of the extraction buffer solution, where the pH was adjusted to 9.8, 9.2, 8.6, and 8.0, respectively), 8nM biotinylated fish primer, and 100µl/ml Protease (Qiagen 7.5U). All lyophilized primers used for hybridization (=fish primers, Table 2-2) were reconstituted to 0.1nmol/µl with 10mM Tris pH 7.4 and stored at –20 ℃ until usage. Aliquots of extraction reagents were kept frozen (-20℃), thawed to room temperature for daily usage, and only used once in order to avoid day-to-day contamination. When more than 1 fish primer was used, the concentration of each fish primer was the same (8nM).

Table2-2 Fish primer sequence

Fish primer designation	Fish primer sequence
Exon *D7b*-fish1	GTGACCCACATGCCATTGCCGGCTCCGACGGTATC
Exon *D7b*-fish2	TGCTGGGGATTCCCCACAGCTCCATCATGGGCTACAA
Exon *D7b*-fish3	AGTGTTAAGGGGATGGGGGGTAAGCCCAGTGACCCAC
Exon *D7b*-fish4	GTCCACAGGGGTGTTGTAACCGAGTGCTGGGGATTCC
Exon *D7b*-fish5	TGGAGAGGTGATAAATCCATCCAAGGTAGGGGCTGGACAG
Exon *D7b*-fish6	GGTGAGCCTTAGTGCCCATCCCCCTTTGGTGGCC

Binding buffer

The binding buffer was composed of 17 mM Tris-HCl (pH 7.4), 8ug/ml Dynabeads M-280 streptavidin (2.8±0.2 µm diameter; Dynal, Oslo, Norway), and 1mM DTT (Sigma, München, Germany).

Washing buffer

The washing buffer was composed of 17 mM Tris-HCl (pH7.4).

2.3.2 Protocol for the separation of fetal DNA from plasma using hybridization capture

The detailed experiment process was optimized as follows [Fig.2-3]:

Step 1: 500 µl plasma sample was added to 554 µl extraction buffer solution (Protease +biotinylated fish primers) in a tube.

Step 2: The suspension solution in the tube was incubated at 60ºC for protein denaturation for 20 mins.

Step 3: Incubate at 95ºC for 20 mins for the denaturation of protease.

Step 4: During this hybridization step the tube was incubated at 60ºC again for 30 mins. Since the fish primers were short and abundant, the result of the competitive hybridization was that the fish primers hybridized with the fetal DNA fragments.

Step 5: After annealing, 1500 µl binding buffer (including magnetic particles) was added to the tube.

Step 6: Incubate at room temperature for 40mins. Because there was streptavidin on the surface of magnetic particles and the streptavidin binds strongly with biotin, the biotinylated fish primers bind to the magnetic particles.

Step 7: The magnetic tip covered with the plastic mantle was moved slowly into the tube. The particles, which have bound DNA, were adsorbed to the surface of the plastic mantle from the binding solution.

Step 8: The magnetic tip, which absorbed magnetic particles, was removed from the tube slowly and transferred into another containing 1500 µl washing buffer.

Step 9: The magnetic beads were moved out of this washing tube and transferred to the PCR tube, which contained the PCR mixture.

Step 10: The magnetic tip was removed and the plastic cover was still in the PCR tube. After about 1min, the magnetic particles with DNA were transferred into the PCR reaction mixture.

Step 11: The PCR tube microplate was heat-sealed and placed in the thermocycler.

Step 12: The PCR reaction started at 50°C for 2 min followed by 95℃ for 10 min. The mixture was thermocycled 50 times between 95℃ for 15s, and 60℃ for 60s.
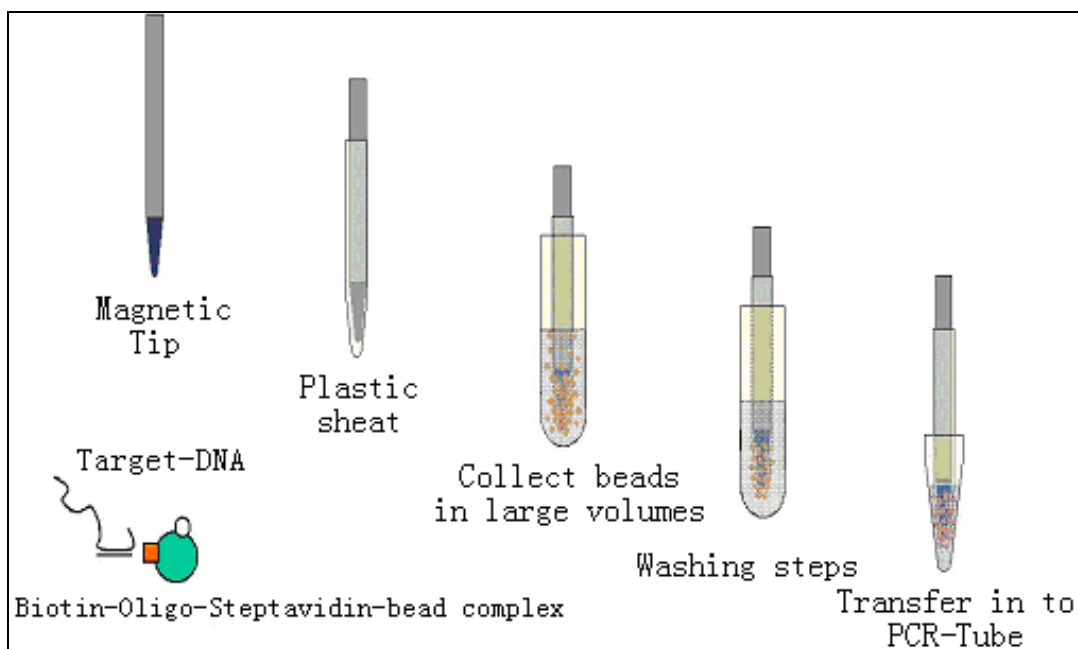
Fig.2-3 The procedure of the method of hybridization capture and magnetic particles

2.3.3 Optimization of the hybridization capture method

2.3.3.1 Different numbers of fish primers

In order to study the influence of the numbers of the fish primers on the efficiency of the DNA extraction, 3 experiments were designed with different amounts of fish primers: A, only 1 fish primer; B, 2 fish primers; and C, 4 fish primers. In all 3 experiments, the total concentrations of the fish primers were the same.

The mean Ct values obtained from PCR in 3 different conditions, described above, were compared using the Independent-Samples T test. A global P-value of <0.05 was considered significant.

2.3.3.2 Different location of fish primers

In order to study the influence of the location of the fish primers on the efficiency of the DNA extraction, 3 experiments were designed with different locations of the fish primers. Fish 1, the fish primers overlap with the amplification primers; Fish 3, the fish primers were 46bp from the

amplification primers; and Fish 5, the fish primers were 201 bp from the amplification primers. In all 3 experiments, the total concentrations of the fish primers were the same.

The mean Ct values obtained from PCR in 3 different locations of fish primers, described above, were compared using Paired-Samples t-test. A global P-value of <0.05 was considered significant.

2.3.3.3 Modification of fish primers

In order to study the influence of fish primers on real-time PCR sensitivity and specificity, 3 experiments were designed. [Fig. 3-9].

Experiment 1

PCR was started without the addition of amplification primers. Thus the ability of 5′-biotinylated fish primer to achieve prime amplification was assessed.

Experiment 2

Biotinylation of the 3`end should prevent amplification. The ability of 3′ and 5′-bis-biotinylated fish primers to achieve prime amplification was assessed in this experiment.

Experiment 3

In order to further study whether the fish primers operate in the amplification of PCR, the amplification primers were replaced by the fish primers in the PCR. Genomic DNA samples were tested as target nucleic acid using the biotinylated fish primers. In the PCR, 5'-biotinylated and 3′-5′-bis-biotinylated fish primers were applied in 6 experiments, respectively.

2.3.3.4 Different concentrations of fish primer

Since the manufacturer recommends that the amount of magnetic particles should not be more than 25mg per PCR, the approximate range of the concentration of the fish primers was calculated. Four groups of experiments were designed with four concentrations (4nM, 8nM, 16nM and 32nM) to optimize this factor. The mean Ct values obtained from PCR in 4 experiments with different concentration of fish primers, described above, were compared using One-Way ANOVA. A global p-value of <0.05 was considered significant.

2.3.3.5 Different pH values

In order to optimize the pH value of the extraction buffer, a set of experiments was designed to study the influence of pH values. In these experiments, the pH of the extraction buffer solution was adjusted to 9.8, 9.2, 8.6, and 8.0 respectively by 0.5M NaOH solutions.

The mean Ct values obtained from PCR in the 4 experiments with different pH values, described above, were compared using One-Way ANOVA. A global p-value of <0.05 was considered significant.

2.4 Automated high-throughput fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

2.4.1 Automated liquid handling

In order to achieve the high-throughput of this method, a special cover for magnetic tips was designed, with a closed top. It can capture and move magnetic particles. A special handle with a magnetic stick was designed. This handle can grasp the special tips so that the magnetic particles can be captured. A special rack adapter was designed which can move magnetic particles from a special tip into PCR tubes. At the same time, in order to improve efficiency, 4 of the 8 handles of the RSP 200/8 were modified while 4 handles remained for pipetting liquids.

The pipetting steps were optimized and the software program was modified, it can be referred to in the appendix.

The optimized detailed experiment process was as follows (see Fig. 2-4):

Step 1: 500 µl plasma sample was pipetted automatically into 554 µl extraction buffer solution (Protease, biotinylated fish primers) in a tube.

Step 2: The tube with suspension solution was moved into an incubator and incubated at 60ºC for protein denaturation for 20 min.

Step 3: Subsequently, the incubation temperature was changed to 95ºC for DNA denaturation for 20 mins. In this period, the protease was denatured.

Step 4: Then the incubation temperature was changed to 60ºC again for the annealing for

30 min. Since the fish primers were short and abundant, the result of the competitive hybridization was that the fish primers hybridized with the fetal DNA fragments.

Step 5: After cooling for 5 min, 1500 ml binding buffer (including magnetic particles) was pipetted automatically into the tube.

Step 6: Then the tube was incubated at room temperature for binding for 40min. Because there was a lot of streptavidin on the surface of the magnetic particles and the streptavidin could be combined strongly with the biotin, the biotinylated fish primers would combine with the magnetic particles.

Step 7: The handle with the magnetic tip held the special tip and was moved slowly into the tube. The particles, which have bound a lot of DNA, were absorbed onto the surface of the special tip from the binding solution.

Step 8: The handle with the special tip, which absorbed many of the particles with the target DNA, was removed from the tube slowly and transferred into another tube with some washing buffer.

Step 9: The handles with the special tip were moved out of this washing tube and transferred to the PCR tube, which contained the PCR mixture.

Step 10: The handle with the magnetic tip was removed and the special tip remained in the PCR tube. After about 1min, the magnetic particles with DNA were put into the PCR reaction mixture.

Step 11: The PCR tube micro-plate was moved to a lower position. Then the PCR tube was detached from the special tip, heat-sealed and placed into the thermocycler.

Step 12: The PCR reaction started at 50°C for 2 min followed by 95℃ for 10 min. The mixture was thermocycled 50 times between 95℃ for 15s, and 60℃ for 60s.

2.4.2 Optimization of automated liquid handling and pipetting steps

Since there are some differences between the detailed procedures of my methods and the commercially available handling robot, the software program of RSP Genesis had to be adapted.

2.4.3 Evaluation of different DNA extraction methods to enrich fetal DNA

At first, 8 pooled plasma samples were prepared. Each sample pool was divided into 2 aliquots.

One of them was extracted using the DSP Virus Kit, and the other was extracted using the manual HCMP method. Secondly, *RHD* was detected for calculating the fetal DNA amount, and *RHCE* was detected for calculating the total DNA using sense primer CTCCGTCATGCACTCCAT, antisense primer TGCCGTTCCAGACAGTATG and probe CAGCAGCACAATGTAGGTGATCTCTCCA. Thirdly, according to the amounts of fetal DNA and total DNA, the percentage of fetal DNA in the total DNA could be calculated. The extraction and enrichment efficiency of the 2 methods was compared and analyzed using the Paired Samples Test.

2.4.4 Probit analysis of optimized nucleic acid extraction methods

The amount of fetal DNA in maternal plasma was calculated from a curve using human DNA as the standard. In order to evaluate the sensitivity of fetal DNA separation from maternal plasma using the optimized DSP Virus Kit method and the optimized manual hybridization capture method, the 95% detection limit was determined. In these experiments, maternal plasma pools with defined concentrations of fetal DNA were diluted on a gradient basis (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256). Each concentration was tested in 8 replicates. The results were analyzed using the calculation procedure, Probit Analysis.

2.5 Evaluation of the optimized free fetal DNA extraction methods in an international survey

I organized an international survey with the co-operating laboratory, Sanquin, in Amsterdam, the Netherlands to compare the methods optimized in this thesis with the methods in other European laboratories specializing in the extraction of free fetal DNA from maternal plasma.

2.5.1 Samples distributed

Samples were prepared by Sanquin as follows: Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood from 252 *RhD*-negative pregnant women previously undergoing red cell antibody screening during weeks 28-30, blood samples that otherwise would have been discarded, were used for the survey. Individual plasma samples were centrifuged for 10 min at 1200*g* without brake and the plasma supernatant centrifuged for a second time for 20 min at 2400*g* without brake. All plasma samples were screened individually for fetal *RHD* sequences

using the Magna Pure LC system (Roche, Basel, Switzerland) for DNA extraction and the real-time PCR protocol as described below. Plasma samples were grouped in three pools according to the Ct values. Eighty-six samples with ct values ranging from 37.01 to 40.91 were grouped in pool 3 (240 ml), which contained a relatively low concentration of cff DNA. Pools 1 (112 ml, n=60, ct 30.41 - 35.49) and 2 (240 ml, n=106, ct 35.50 - 36.99) showed higher concentrations. The three plasma pools from RhD-negative pregnant women carrying RhD-positive fetuses, the genomic DNA standards (500 pg/µl, 50 pg/µl and 5 pg/µl), real-time-PCR protocol, primers and probes for *RHD* exons 5 and 7 were sent from one centre to 12 partners of the SAFE NoE and 1 company (QIAGEN, Hilden, Germany).

2.5.2 DNA extraction

Each laboratory (lab) used its own protocol for DNA extraction, and if more than one method was available in one lab, each DNA extraction protocol was applied. Each pool was sent in three aliquots and tested three times in three different runs in order to avoid false conclusions due to run-to-run variations. Many labs performed replicate testing on each aliquot, although this was not an obligatory requirement. Within this survey the author evaluated three methods: optimised DSP Virus Kit, manual and automated hybridisation capture and magnetic particles.

The protocol of the DSP Virus Kit method was the same as described in Chapter 2 of Materials and Methods. The protocols of manual and automated hybridisation capture and magnetic particles methods were the same as those in Chapter 3 and Chapter 4 of Materials and Methods, except that fish primers for *RHD* exon 5 were added to detect the status of *RhDψ* (Table 2-3). In order to extract fetal DNA, four fish primers were used. Two were for exon 7 and the other two were for exon 5. The total concentration of the fish primers on exon 7 and exon 5 were 8 nM .

Table2-3 Fish primers sequence for *RHD* exon 5 and 7 used in the survey

Fish primer designation	Fish primer sequence
Exon D7-fish1	GGAATATGGGTCTCACCTGCCAATCTGCTTATAATAACACT TGTCCA
Exon *D7*-fish2	TGTTAAGGGGATGGGGGGGTAAGCCCAGTGACCCACATGCC
Exon *D5*-fish1	GCAGGAGTGTGATTCTGGCCAACCACCCTCTCTGGCC

Exon *D5*-fish2	CCCTGAGATGGCTGTCACCACGCTGACTGCTACAGCATAGT AGG

2.5.3 *RHD* real-time PCR protocol

The *RHD* specific exon5/exon7 duplex PCR (50 µl reactions) consisted of 1x Mastermix containing polymerase dNTPs, buffer and MgCl$_2$ (Universal Mastermix, Applied Biosystems, Foster City, CA), primers and probe (Tables 2-4) and 15 µl template. After initial incubation at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, 50 two-step cycles at 95°C for 15 seconds and 60°C for 60 seconds were performed. The *RHD* concentrations of plasma pools 1-3, which were obtained in different labs, were calculated centrally, based on the ct values of pools and each standard curve, respectively, using the equation $10^{\text{(intercept-Ct)/slope}}$.

Table 2-4: Primers for *RHD* real-time PCR used in the survey

Primer designation	Primer sequence	Label	*RHD* region
RHD 940S[1]	GGGTGTTGTAACCGAGTGCTG	none	exon 7
RHD 1064R[1]	CCGGCTCCGACGGTATC	none	exon 7
RHD 968T	CCCACAGCTCCATCATGGGCTACAA	FAM-TAMRA	exon 7
RHD ex5F[2]	CGCCCTCTTCTTGTGGATG	none	exon 5
RHD ex5R[2]	GAACACGGCATTCTTCCTTTC	none	exon 5
RHD ex5T[2]	TCTGGCCAAGTTTCAACTCTGCTCTGCT	VIC-TAMRA	exon 5

[1]Faas 1998

[2]Finning 2002

2.6. Statistics

All the experimental data was analyzed using the SPSS software 10.0. Details of the statistical method applied are provided in the description of each experiment.

Chapter 3   **Results**

3.1 Optimization of the QIAamp DSP Virus Kit for the extraction of fetal DNA from maternal plasma

3.1.1 Comparison between with carrier RNA and without carrier RNA
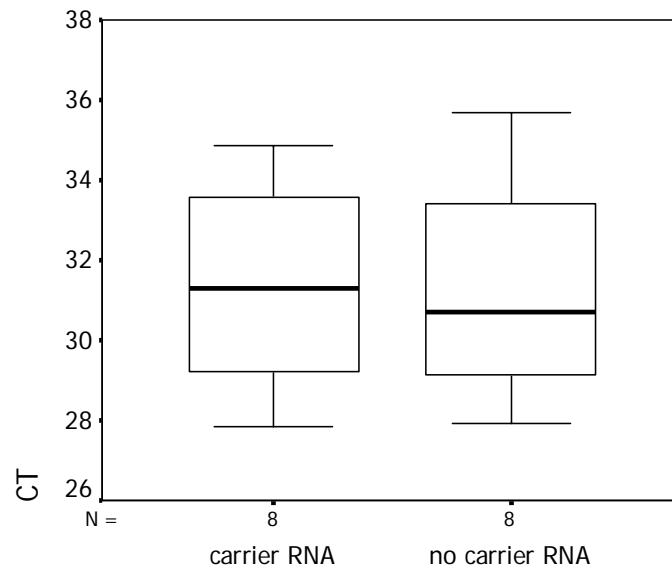
Fig.3-1 Influence of carrier RNA

As shown in Fig.3-1, the mean Ct was 31.36 when carrier RNA was added, and the mean Ct was 31.27 without carrier RNA. There was no significant influence of carrier RNA (p=0.593, n=8).

3.1.2 Difference between 75μl protease and 100μl protease

As shown in Table 3-1, when the plasma samples were denatured with 100μl protease, 22 plasma pools passed the membrane within 10min, and the remaining 2 plasma pools passed through in 10-15min(12mins, 13mins). When the plasma samples were denatured with 75μl protease, 21 plasma pools passed the membrane within 10mins, and the remaining 3 cases passed through in more than 15mins (18mins, 21mins, 23mins)

Table 3-1, the result of the time of lysate passing through the membrane

Time of passing through the membrane	<10min	10-15min	>15min
No. of cases in 100μl protease	22	2	0
No. of cases in 75μl protease	21	0	3
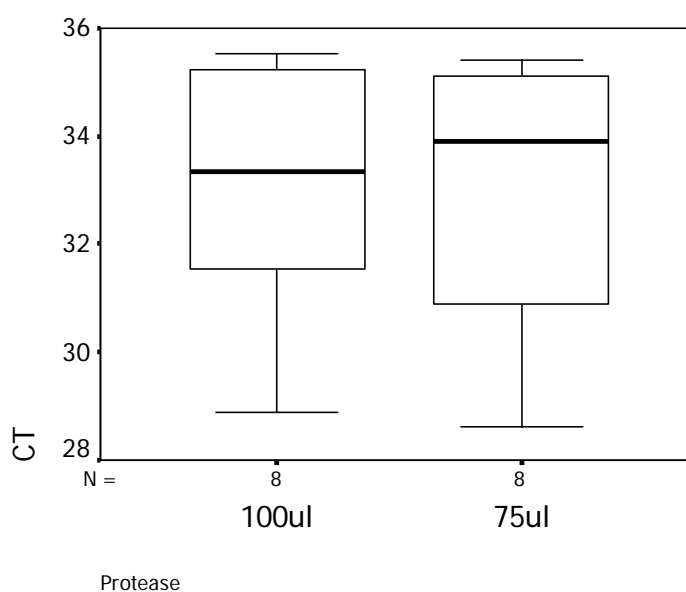
Fig. 3-2 The optimization of the amount of protease

There was no significant difference between Ct values when comparing 75 μl protease and 100 μl protease, respectively (Fig. 3-2, p=0.827, n=8). The mean Ct was 33.07 when 100 μl protease was applied, and the mean Ct was 32.99 when 75 μl protease was used..

3.1.3 Comparison between the DNA elution in water and in AVE buffer

Eluting fetal DNA in water resulted in lower Ct-values than eluting fetal DNA in AVE buffer (Fig. 3-3,$p<0.05$, n=14). The mean Ct was 32.17 when the DNA was eluted in water, and the mean Ct was 33.41 when the DNA was eluted in AVE buffer.
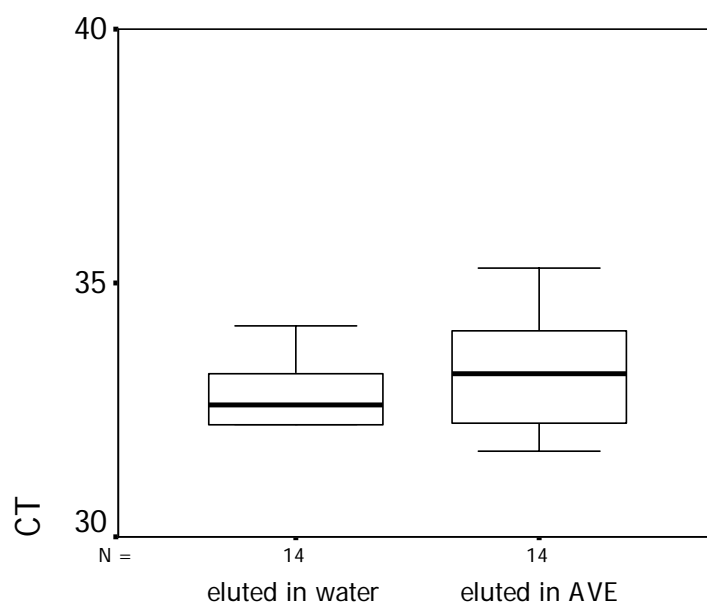
Fig. 3-3 Comparison between the DNA elution in water and in AVE buffer

3.1.4 The 95% detection limit analysis

The 95% detection limit of the optimised protocol calculated with the probit analysis was 138 pg/ml (21geq/ml, Fig.3-4). When the concentration of DNA was more than or equal to 179.3 pg/ml, all the results were positive. When the concentration of DNA was 89.67pg/ml, two of the eight samples were false negative, thus the hit-rate was 75%.
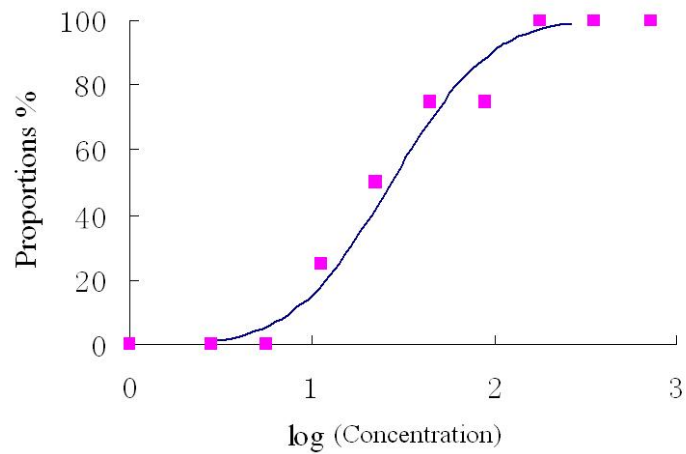
Fig 3-4 95% Detection limit analysis.

Table 3-2. Results of the 95% detection limits in the Probit Analysis

Concentration of DNA (pg/ml)	717.3	358.7	179.3	89.7	44.8	22.4	11.2	5.6	2.8	0
Results	8/8	8/8	8/8	6/8	6/8	4/8	2/8	0/8	0/8	0/8
Positive rate	100	100	100	75	75	50	25	0	0	0

3.2 Fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

3.2.1 Optimization of fish primers

3.2.1.1 Different number of fish primers
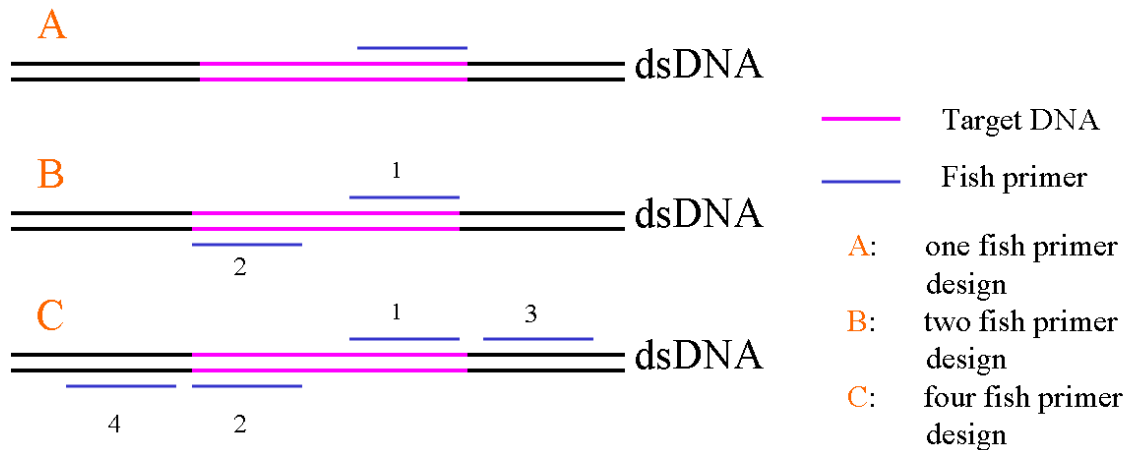
Fig.3-5 Different numbers of fish primers

In order to study the influence of the number of fish primers on the efficiency of the DNA extraction, 3 experiments were designed with different numbers of fish primers: A 1 fish primer, B 2 fish primers; and C 4 fish primers (Fig.3-5).

As shown in Fig.3-6, the mean Ct-values of 1-fish-primer design, 2-fish-primer design, and 4-fish-primer design were 36.6, 35.1, and 34.3 respectively. The 2-fish-primer design was significantly better than the 1-fish-primer design (p=0.014), but there was no significant difference between the 2-fish-primer design and the 4-fish-primer design (p>0.05). Two fish primers were applied in further experiments.
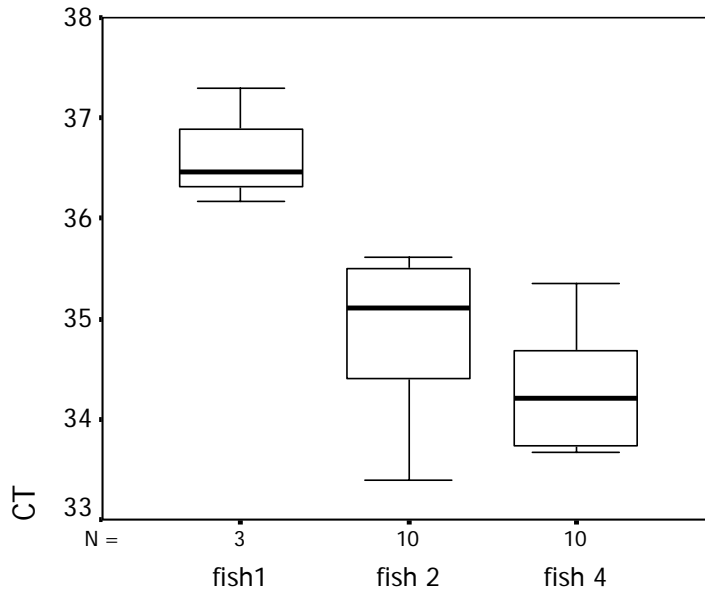
Fig.3-6 Different number of fish primers

3.2.1.2 Different location of fish primers

In order to study the influence of the location of the fish primers on the efficiency of the DNA extraction, 3 experiments were designed with different locations of the fish primers (Fig.3-7). Fish 1, the fish primers overlap with the amplification primers; Fish 3, the fish primers were 46bp from the amplification primers; and Fish 5, the fish primers were 201bp from the amplification primers.
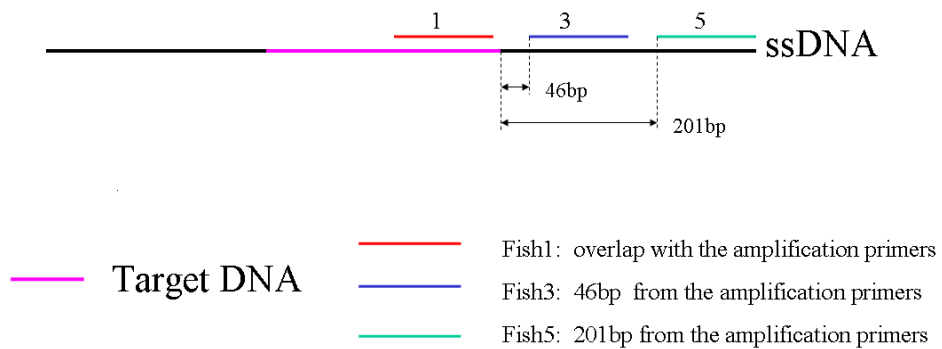
Fig.3-7 different location of fish primers
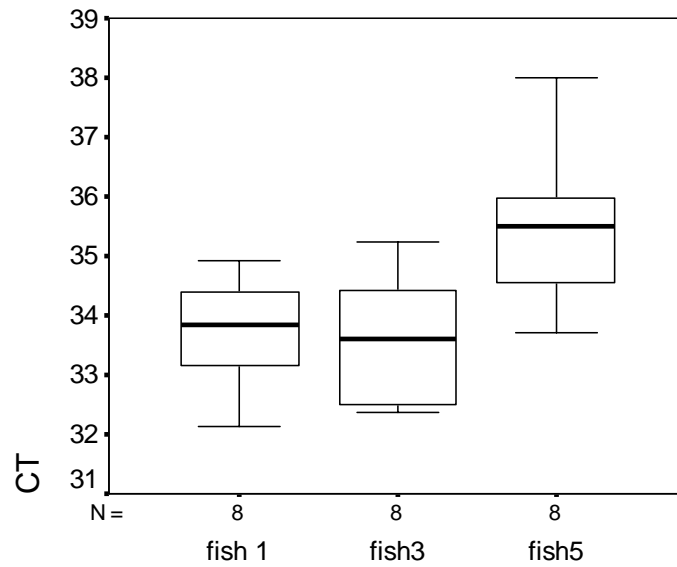
Fig.3-8. Different location of fish primers

The mean Ct was 33.8 when the fish primers overlapped with the amplification primers. The mean Ct was 33.6 when the fish primers were 46bp from the amplification primers. And the mean Ct was 35.3 when the fish primers were 201bp from the amplification primers (Fig.3-8). The Ct values of Fish-1 and Fish-3 experiments were significantly lower than those of Fish-5 ($p<0.01$), and there was no significant difference between those of Fish-1 and Fish-3 ($p>0.05$).

3.2.1.3 The optimization of biotinylation of the fish primers

Since all of the fish primers were transferred into the PCR tube in this method, the fish primers probably will work in the amplification of PCR and thus decrease specificity. In order to study the influence of fish primers in the amplification, 3 experiments were designed. [Fig. 3-9].
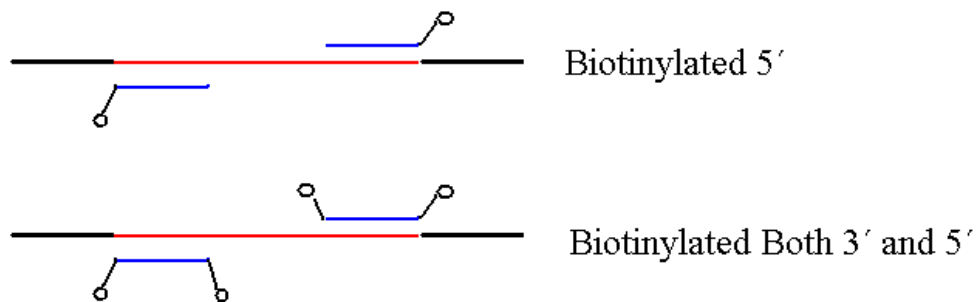
Fig.3-9 Mono-biotinylation and bis-biotinylation of fish primers

Experiment 1:

The DNA samples used here were isolated by the hybridization capture of target nucleic acid with the biotinylated fish primers. Since all of the fish primers were transferred into the PCR tube in this method, the amplification primers were not added into the PCR mixture. The fish primers were biotinylated on 5'only.

Table 3-3 Results of experiment 1

	Sample 1	Sample 2	Sample 3	Sample 4	NC[1]
With amplification primers in PCR	38.5	37.5	36.8	36.4	50
Without amplification primers in PCR	44.2	45.9	37.6	45.7	50

[1]NC: negative control=water

As shown in table 3-3, the real-time PCR results of the 4 plasma pools were positive when the amplification primers were added to the PCR mixture. However, when the amplification primers were not added into the PCR mixture, the results of the 4 samples were also weak positive. Obviously, these results were false positive.

Experiment 2:

Apart from the fact that the fish primers were biotinylated on both 3' and 5', the other details of the experiment were the same as in experiment 1.

Table 3-4 Results of experiment2

	Sample 1	Sample 2	Sample 3	Sample 4	NC
With amplification primers in PCR	35.9	36.8	35.0	34.6	50
Without amplification primers in PCR	50	50	50	50	50

As shown in Table 3-4, when the fish primers were biotinylated on both ends, 3' and 5', the results of the 4 samples were positive when the amplification primers were added into the PCR

mixture; the results of the 4 samples were all negative when the amplification primers were not added into the PCR mixture.

Experiment 3:

In order to further study whether the fish primers operate in the amplification of PCR, the amplification primers were replaced by the fish primers in the PCR. In this experiment genomic DNA was used as the template and no hybridization was performed before the beginning of PCR.

Table 3-5 Results of experiment 3

Samples	5' biotinylated	3' and 5' bis-biotinylated
1	37.3	47.8
2	36.1	50
3	30.6	50
4	30.9	50
5	32.2	50
6	32.2	50
NC	50	50

When 5´-only-biotinylated fish primers were used in PCR the results from 6 samples were all positive (Table 3-5). However, when the fish primers were biotinylated on both ends 3' and 5', the results of the 6 samples were negative, except in sample 1 which showed a very high Ct-value.

3.2.2. Optimization of hybridization condition

3.2.2.1 Different concentration of fish primer:

32nM fish primers (mean ct 35.7) were worse than 4nM (mean ct 34.4), 8nM (mean ct 34.5) and 16nM (mean ct 34.7) ($p<0.01$, Fig.3-10); 8nM fish primers were slightly better than 4nM and 16nM (not significant).
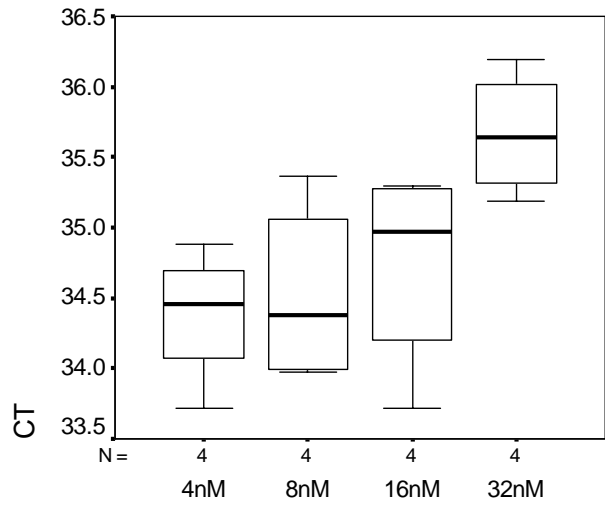
Fig. 3-10 results of different concentration of fish primer

3.2.2.2 Different pH values:

In the experiments, four pH values of the extraction buffer solution were studied: 9.8, 9.2, 8.6, and 8.0. The mean Ct values were 35.2, 34.3, 35.3, and 35.8 respectively. The optimal pH for hybridization was 9.2. There are significant differences between the Ct values of pH=9.2 and 9.8, 9.2, 8.6, 8.0., $p < 0.02$, Fig.3-11.
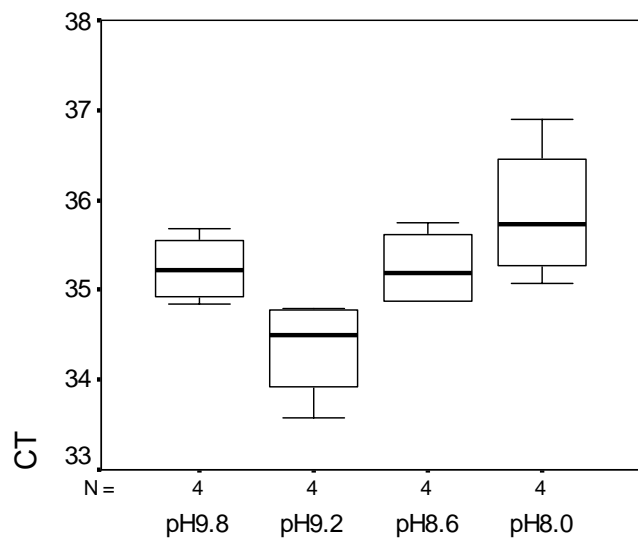
Fig.3-11 Results of different pH values

3.2.3 The 95% detection limit

The results of the Probit Analysis are shown in Table 3-6 and Fig. 3-12. The results implied that when the concentration of DNA was more than or equal to 358.6pg/ml, all the results were positive. When the concentration of DNA was 179.3pg/ml, one of the eight samples was false negative, and so the positive rate was 87.5%. The 95% detection limit for the hybridization capture method was 358pg/ml (54geg/ml).

Table 3-6. Results of the 95% detection limits in the Probit Analysis

Concentration of DNA (pg/ml)	717.3	358.6	179.3	89.6	44.8	22.4	11.2	5.6	2.8	0
Results	8/8	8/8	7/8	6/8	5/8	3/8	4/8	4/8	0/8	0/8
Positive rate	100	100	87.5	75	62.5	37.5	50	50	0	0
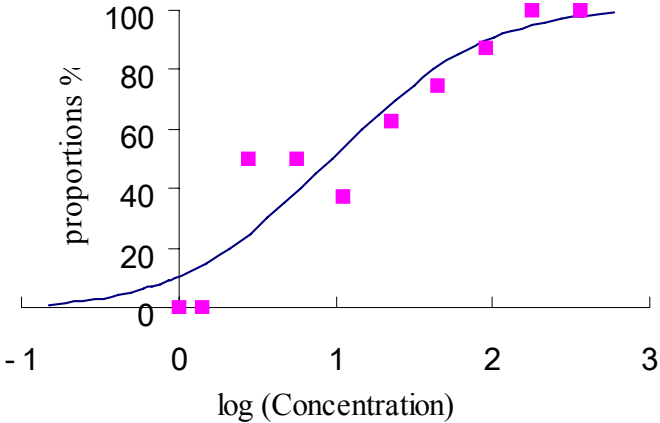
Fig.3-12 Probit Analysis of the HCMP method

3.2.4 The evaluation of the efficiency of fetal DNA extraction and enrichment in hybridization capture and magnetic particles

The mean percentages of fetal DNA (calculated from *RHD*) in total DNA (calculated from *RHCE*) extracted by HCMP and the non-specific extraction method (DSP Virus Kit, Qiagen) in 8 different plasma pools were 4.83 % and 2.86 % respectively (Fig.3-13). The extraction and enrichment efficiency of the HCMP method is significantly better than the non-specific method ($p<0.05$, n=8).
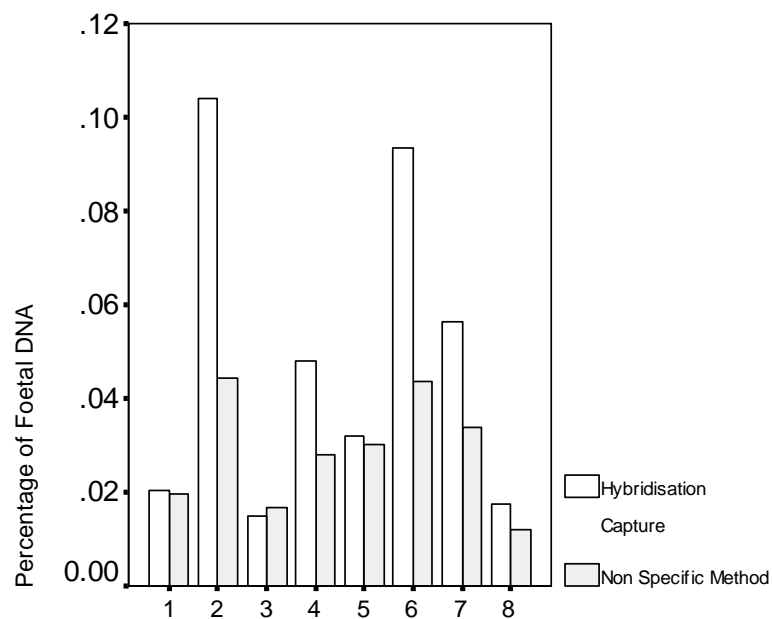
Fig.3-13 the evaluation of extraction and enrichment efficiency of fetal DNA

3.3 High-throughput fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

3.3.1 Optimized protocols

A commercially available liquid handling robot (RSP Genesis 200/8; Tecan, Crailsheim, Germany) was modified in some details to perform particles transfer and apply the magnetic tips.

Firstly, a special tip cover was designed with a closed top (Fig. 3-14-A). It can capture magnetic particles on its outside surface when there is a magnetic stick inside. When the magnetic stick is removed, the captured magnetic particles are released.

Secondly, a special handle with a magnetic stick (Fig. 3-14-B) was designed. This handle can grasp the special tips to capture the magnetic particles in a hybridization solution, and dispose the special tips after the tips are moved into the PCR tube, then the magnetic particles are released (Fig.3-14-C).

Thirdly, a special rack adapter (Fig. 3-14-D) was designed. The top part of this adapter is a tip container. Its bottom part is a platform, which can contain the PCR micro-plates. The platform can be moved up and down to 2 fixed positions. When the platform is moved to the upper position, the tips will be immersed into the PCR tubes. When the magnetic stick is removed, the magnetic particles on the outer surface of the tips will be positioned in the PCR tubes. Then, the platform will be moved to the lower position and the tips will be moved out of the tubes. The PCR micro-plated can be moved out of this adapter, heat-sealed with foils and placed into the thermocycler.

In order to improve the efficiency, 4 of the 8 handles of the RSP 200/8 were modified to be used for moving magnetic particles (Fig.3-14-E). These special handles were used to capture the magnetic particles, and the other 4 handles were still used for liquid handling. The modified pipetting workstation is shown in Fig.3-14-F.
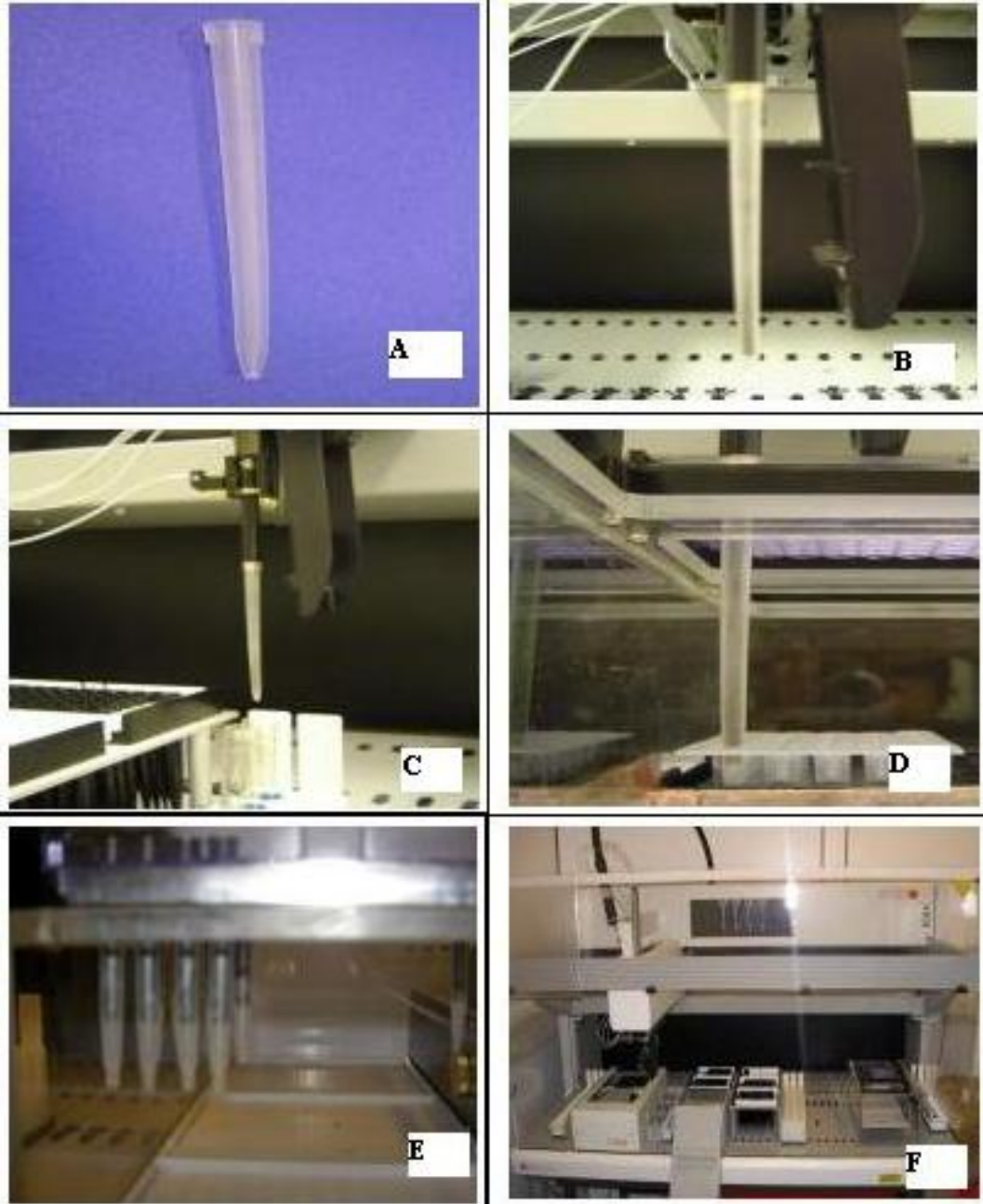
Fig.3-14 High-throughput fetal DNA extraction with hybridization capture and magnetic particles

3.3.2 The time required for automated HCMP

When only one sample was tested, the total time required to perform the whole procedure was 259.1min (Table 3-7, 119 min without PCR). When a batch of samples (96 samples) was tested, this took 335.9min (196 min without PCR). In the automated procedure, a lot of time would be saved, mainly in incubation, when many samples were tested. The efficiency of the test can therefore be greatly increased.

Table 3-7 Protocol for automated extraction and amplification (unit: min)

	Protocol	single test time			96 tests time		
		operator	running	total	operator	running	total
1	Dispense extraction buffer into tube.	0.1			9.6		
2	add 500µl sample and mix.	0.1	0.3		9.6	7.2	
3	incubate at 60°C for 20 min.	0.2	20		0.2	20	
4	incubate at 95°C for 20 min	0.2	20		0.2	20	
5	incubate at 60°C for 30 min.	0.2	30		0.2	30	
6	add 1500 µl Hybridization buffer	0.1	0.3		9.6	7.2	
7	incubate at RT for 40 min.	0	40		0	40	
8	place the tube into Tecan.	0.1			0.1		
9	collect the magnetic particles		1.5			36	
10	seal and centrifuge the plate.	3			3		
11	start real-time PCR	3	140		3	140	
	Total time not including PCR	7	112.1	119.1	35.5	160.4	195.9
	Total time including PCR	7	252.1	259.1	35.5	300.4	335.9

3.3.3 The 95% detection limit of automated HCMP

The results of the Probit Analysis are shown in Table 3-8 and Fig. 3-15. The results implied that when the concentration of DNA was more than or equal to 358.6 pg/ml, all the results were positive. When the concentration of DNA was 179.3 pg/ml, one of the eight samples was false negative, and so the positive rate was 87.5%. According to Table 3-8, the Probit Analysis results were acquired as shown in Fig.3-15. The 95% detection limit for the hybridization capture method was 286 pg/ml (43 geg/ml).

Table 3-8. Results of the 95% detection limits in the Probit Analysis

Concentration of DNA (pg/ml)	717.3	358.6	179.3	89.6	44.8	22.4	11.2	5.6	2.8	0
results	8/8	8/8	7/8	6/8	6/8	3/8	3/8	3/8	2/8	0/8
positive rate	100	100	87.5	75	75	37.5	37.5	37.5	25	0
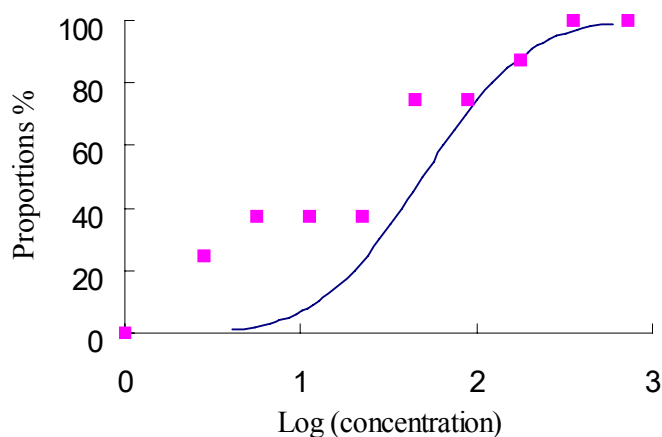
Fig.3-15 Probit Analysis of the HCMP method

3.4 Evaluation of the optimized DSP, hybridization capture in comparison with previously established methods for fetal DNA isolation from maternal plasma

Table 3-9: Detection rate of replicate testing pools 1-3 with different DNA extraction protocols.

lab Code	extraction method	pool 1		pool 2		pool 3	
		exon 7	exon 5	exon 7	exon 5	exon 7	exon 5
1	DSP‡	3/3	3/3	3/3	3/3	3/3	3/3
2	HP	3/3	3/3	3/3	3/3	3/3	3/3
3	MINI	3/3	3/3	3/3	3/3	3/3	3/3
4	MINI	3/3	3/3	3/3	3/3	3/3	3/3
5	MINI	3/3	3/3	3/3	3/3	3/3	3/3
6	MINI	3/3	3/3	2/3 (3/3)[†]	3/3 (3/3)	2/3 (3/3)	3/3 (3/3)
7	MINI	3/3	2/3	3/3 (3/3)	3/3 (3/3)	2/3 (3/3)	2/3 (3/3)
8	MINI	3/3	3/3	3/3 (3/3)	3/3 (3/3)	1/3 (3/3)	2/3 (3/3)
9	MINI	n.t.	n.t.	3/3	3/3	3/3	3/3
10	CST	3/3	3/3	3/3	3/3	3/3	3/3
11	M-HCMP	3/3	3/3	3/3	3/3	3/3	3/3
12	MIDI	3/3	3/3	0/3	0/3	0/2	1/3
13	MP	2/2	2/2	2/2	2/2	2/2	2/2
14	MP	3/3	3/3	3/3	3/3	3/3	3/3
15	A-HCMP	3/3	3/3	3/3	3/3	3/3	3/3
16	MDx	3/3	3/3	3/3	3/3	3/3	3/3
17	M48	3/3	3/3	3/3	3/3	3/3	3/3
18	EZ1	3/3	3/3	3/3	3/3	3/3	3/3

‡ DSP: Optimized QIAamp DSP Virus Kit; HP: High Pure PCR Template Preparation Kit, MINI: QIAamp DNA blood Mini Kit; CST: CST genomic DNA purification kit, MIDI: QIAamp DNA blood Midi Kit, MP: MagnaPure LC; M-HCMP: Manual hybridization capture and magnetic particles, A-HCMP: Automated hybridization capture and magnetic particles, MDx, M48 and EZ1 are nucleic acid separation workstations from Qiagen.

†The DNA extraction procedures 6-8 were repeated after review of the protocols and application of an improved protocol for the DNA Blood Mini Kit. The results of this second round of the survey are shown in brackets.

Table 3-10: Quantification of D-positive fetal DNA using *RHD* exon 7 primers for amplification after manual DNA extraction

exon 7	1*	2	3	4	5	6	7	8	9	10	11	12
Extraction Method†	DSP	HP	MINI	MINI	MINI	MINI	MINI	MINI	MINI	CST	M-HCMP	MIDI
Detection System	ABI 7700	ABI 7000	ABI 7700	ABI 7000	ABI 7000	ABI 7700	ABI 7500	ABI 7700	ABI 7000	ABI 7300	ABI 7700	ABI 7700
plasmavol. [mL]	0.5	0.8	0.8	1	0.4	0,4 [1]‡	0,8 [1]	0,4 [1]	1	1	0.5	1
elutionvol. [microL]	20	50	50	50	50	50 [60]	55 [60]	60	65	50	2	50
pool 1	1492	267	376	365	194	105	207	71	n.t.	239	320	24
[pg/well]	1054	276	423	407	171	149	2	101	n.t.	184	218	37
	1420	205	536	433	229	131	244	58	n.t.	198	241	18
pool 2	220	54	19	19	15	0 [9]	pos. [6]	pos. [27]	59	44	91	0
[pg/well]	184	51	20	pos.	14	pos. [10]	pos. [30]	pos. [23]	50	24	37	0
	208	32	29	39	36	pos. [16]	pos. [18]	6 [pos.]	69	38	27	0
pool 3	159	pos.	pos.	10	6	11 [pos.]	4 [9]	0 [pos.]	45	22	77	0
[pg/well]	80	pos.	pos.	50	12	pos. [10]	pos. [24]	0 [pos.]	38	9	13	0
	96	pos.	35	pos.	pos.	0 [9]	0 [13]	pos. [pos.]	33	21	14	n.t.
pool 1	3979	1111	1565	1216	1614	876	950	588	n.t.	796	640	98
[pg/ml]	2812	2296	1761	1357	1428	1242	10	843	n.t.	613	437	150
	3786	1709	2235	1442	1909	1092	1119	487	n.t.	660	482	71
pool 2	586	225	78	64	128	0 [35]	pos. [24]	pos. [108]	256	147	182	0
[pg/ml]	491	423	83	pos.	119	pos. [41]	pos. [119]	pos. [93]	217	81	74	0
	556	266	121	130	300	pos. [62]	pos. [72]	52 [pos.]	298	125	55	0
pool 3	424	pos.	pos.	33	53	89 [pos.]	19 [36]	0 [pos.]	194	74	154	0
[pg/ml]	214	pos.	pos.	166	100	pos. [41]	pos. [95]	0 [pos.]	163	30	27	0
	256	pos.	147	pos.	pos.	0 [37]	0 [53]	pos. [pos.]	144	69	28	n.t.

*Arbitrary number for survey participant, †For abbreviations see Table 3-9

‡Results from a second round of the survey are shown in brackets

Table 3-11: Quantification of D-positive fetal DNA using *RHD* exon 7 primers for amplification after automated DNA extraction

exon7 Extraction Method Detection System plasmavol. [mL] elutionvol. [µL]	13* MP† ABI 7000 1 50/ 100‡	14 MP ABI 7000 1 50	15 A-HCMP ABI 7700 0.5 2	16 MDx ABI 7700 0.265 91	17 M48 ABI 7700 0.4 100	18 EZ1 ABI 7700 0.4 100
pool 1 [pg/well]	632 299 n.t.	342 322 405	474 345 203	235 215 235	75 87 78	143 83 96
pool 2 [pg/well]	142 46 n.t.	49 56 64	86 83 51	60 50 pos.	pos. 25 pos.	pos. 14 5
pool 3 [pg/well]	90 31 n.t.	20 27 48	74 21 20	pos. pos. pos.	pos. pos. pos.	pos. pos. pos.
pool 1 [pg/ml]	1684 1995 n.t.	1138 1072 1350	949 689 407	5481 5028 5268	1255 1450 1292	2383 1387 1606
pool 2 [pg/ml]	379 306 n.t.	164 187 214	172 167 102	1379 1145 pos.	pos. 412 pos.	pos. 239 81
pool 3 [pg/ml]	239 207 n.t.	65 89 160	147 43 41	pos. pos. pos.	pos. pos. pos.	pos. pos. pos.

*Arbitrary number for survey participant

†For abbreviations see Table 3-9

‡50µl elution volume on day 1, 100µl on day 2.

3.4.1 DNA extraction methods

At the international survey, the QIAamp DNA Blood Mini Kit (Faas BH et al. 1998; K.M. Finning et al. 2002; YM Dennis Lo et al. 1997) was applied in 8 labs. The High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) (Zimmermann B. et al. 2005), the QIAamp DNA Blood Midi Kit (Qiagen), the CST genomic DNA purification Kit (Invitrogen, Carlsbad, CA) and the Optimization DSP Virus Kit, manual HCMP, were applied as manual methods in one lab each. The MagnaPure LC system (Roche) was used in 2 labs (van der Schoot CE et al. 2004). The BioRobot MDx with the QIAamp Virus MDx Kit, the BioRobot M48 with

the MagAttract Virus Mini M48 Kit, the EZ1 workstation with the EZ1 Virus Mini Kit (all Qiagen) and manual（HCMP) were used as automated methods in one lab each. All but one lab provided a standard curve with a slope between 3.1 and 3.9. One lab using the Light Cycler (Roche) did not fulfil this inclusion criterion. Real time PCR instruments applied by the different labs are listed in Tables 3-9, 3-10 and 3-11.

3.4.2 Results of real-time PCR

In order to draw the correct conclusions from the different labs only data was compared where the slope of the standard curve ranged between 3.1 and 3.9 and the correlation between ct and DNA concentration (R-value) was ≥ 0.98. Furthermore, a concentration of DNA is only given when the delta ct of replicates is ≤ 1.5 ct and the difference between ct (water control) -ct (pool 1-3) is > 1. Lower positive results are marked with "pos.". Several labs performed replicate real time PCR on one extract. If none or only one of three replicates were recorded as positive, the result was filed "negative (0)", if two or three of three replicates were recorded as positive, the result was filed "positive (pos.)".

Fourteen of 18 extraction procedures showed a 100% concordance for the detection of D-positive cff DNA in plasma pools 1-3 (Table 3-9). Water controls showed D-negative results with all methods except with Methods 9 and 12 (1 false positive result with exon 7 primers on 1 of 3 days with each method).

The DNA Blood Midi Kit performed less well than other technologies. Additionally, 3 of the 4 labs, which failed to extract enough cff DNA for reliable amplification and detection used the QIAamp DNA Blood Mini Kit (Method Codes 6-8). Each standard operation procedure from the labs using this kit was reviewed. The omission of the last centrifugation step of the dry column to eliminate ethanol was identified as a potential cause for the poor results, especially from pools 2 and 3. It was also noted that labs 6 - 8 had used less than 500µl plasma for the initial DNA extraction. There was enough plasma from pools 2 and 3 to repeat the DNA extraction including the last centrifugation step of the dry column and improved results were observed in those 3 labs (Method codes 6-8) who participated in this second round of the survey.

Because each lab used three centrally distributed DNA standards, it was possible to determine the quantity of extracted cff DNA in each well and to calculate the yield per ml from each pool (Tables 3-10, 3-11). With the QIAamp DSP Virus Kit the highest yield per ml was obtained (Fig. 3-16), however this method was initially used by one lab only.

The results in the Table 3-10 showed that the optimised DSP and hybridisation capture method had good performance in the experiments. It could be concluded from Table 3-10 that the recovery of the optimised DSP method was much higher than other methods. The results in Table 3-11 showed that the yield of DNA (pg/well) isolated by the hybridisation capture and magnetic particles method was higher than the yield of other methods (Fig. 3-17). In Fig. 3-16, it could also be demonstrated that the yield of the optimised manual DSP method was much higher than the yield of other manual methods. Additionally, the yield of manual HCMP was higher than the yield of other manual methods. In Fig. 3-17 it is shown that the yield of automated HCMP was higher than the yield of other automated methods (except for the yield of MagnaPure LC which was similar to that of the automated hybridisation capture method).

In order to further elucidate this finding, a comparison between the QIAamp DSP Virus Kit and the QIAamp DNA Blood Mini Kit was performed in an independent lab (Sanquin, Amsterdam, the Netherlands) using 27 samples from D-negative pregnant women carrying D-positive fetuses at week 30. This experiment confirmed the higher yield of free fetal DNA of QIAamp DSP Virus Kit in comparison with the QIAamp DNA Blood Mini Kit.

Finally, it is noticed that, although the results were correct, the quantity determined from pool 3 was at the detection limit in those procedures, which used less than 0.5 ml (labs 5, 8, 16-18).
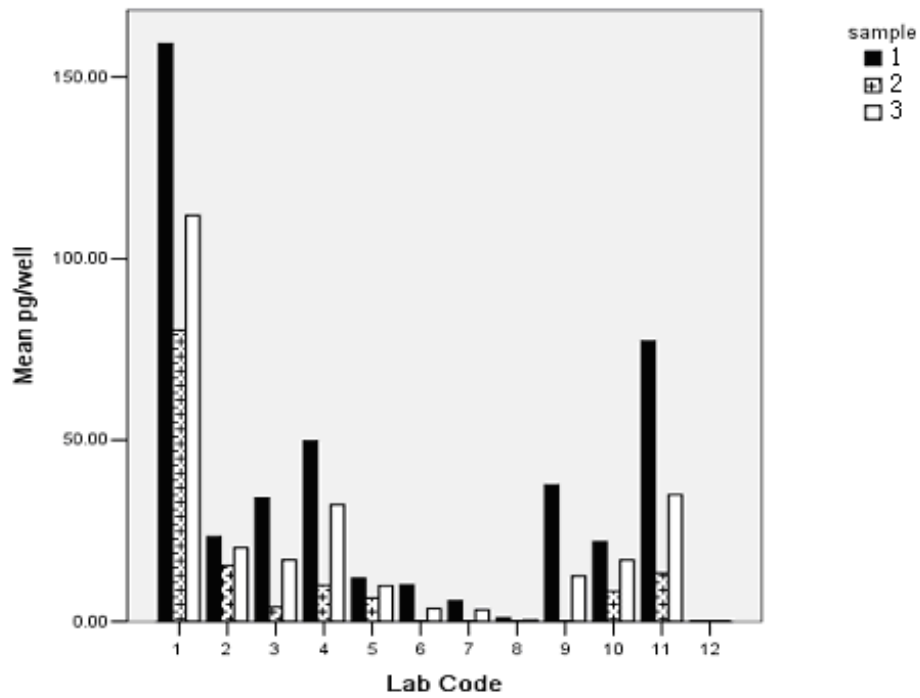
Fig.3-16 Quantification of D-positive fetal DNA using *RHD* exon 7 primers for amplification after manual DNA extraction
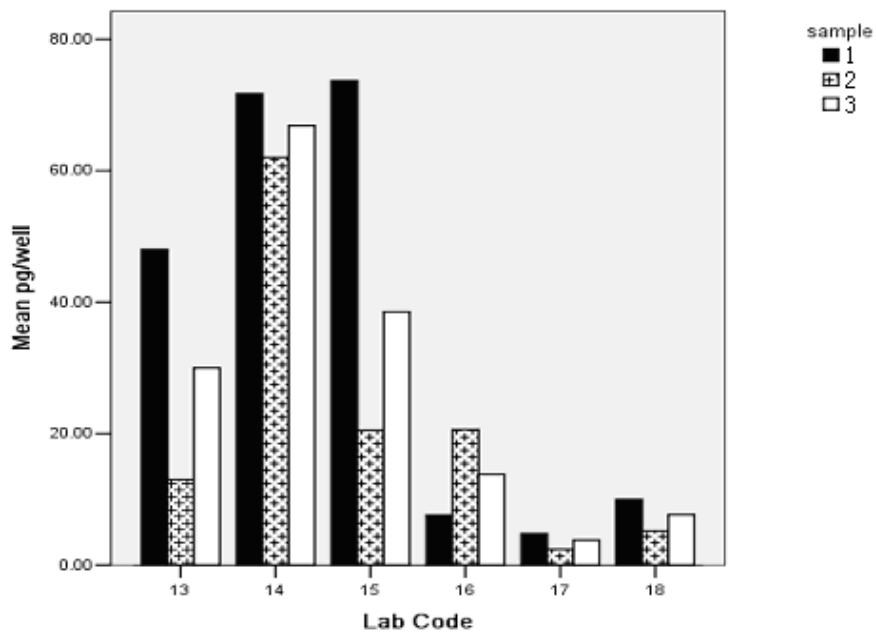

Fig.3-17 Quantification of D-positive fetal DNA using *RHD* exon 7 primers for amplification after automated DNA extraction

Chapter 4   **Discussion**

To prevent anti-D immunization in D-negative pregnant women through transplacental haemorrhage, it has become common practice to offer routine antenatal anti-D prophylaxis (RAADP), usually at 28-34 weeks of gestation, and/or within 72h after delivery. On the other way, anti-D is frequently not indicated. In a predominantly Caucasian population, 15-18 % of all pregnancies occur in D-negative women. In about 40% of all D-negative cases anti-D prophylaxis is not indicated because the fetus is D-negative. In those pregnancies women receive this therapy unnecessarily. Consequently, it will be beneficial if a method (especially a high-throughput method) is developed for determining the fetal D type in all pregnant D- women.

Since the fetal RhD status determination from maternal blood was reported using free fetal DNA in 1997 (Lo YMD, et al. 1997), many researchers began to focus on genotyping for the fetal RhD with the free fetal DNA extracted from the maternal blood. But the diagnostic accuracy of this method is always disputed. Lo YMD. et al reported only 16 of 21 (76.2%) correct fetal RhD genotyping results from maternal blood in their first paper in 1993 using fetal cells (Lo YM, et al. 1993). Bischoff FZ et al. (1999) reported 14 of 20 (70%) RhD-positive fetuses showing a 99 base pair *RHD*-specific PCR product. Yatama MK et al. (1999) reported a sensitivity and specificity of seminested PCR of 81 and 100% respectively for the detection of fetal RhD positivity in the whole blood of pregnant women. Zhang XY et al. (2001) reported 12 of 13 (92%) correct RhD genotyping results using DNA isolated by QIAamp Blood Kit. Randen I. et al. investigated 114 RhD-negative pregnant women for the presence of fetal *RHD*. Fetal DNA was extracted using a QIAamp DNA Blood Mini Kit. The discrepancy between genotyping and serological RhD typing of the babies postpartum was 8%. Finning K et al. (2004) tested 283 pregnancies, of which a correct genotyping result could be confirmed in 223 cases. The method of DNA extraction is QIAamp Blood Mini Kit. Birch L et al. (2005) reported the sensitivity of fetal gender determination in pregnancies to be 99%, with 100% specificity using the QIAamp Blood Mini Kit for DNA isolation. Machado IN et al.(2006) reported an accuracy of 97.3%, a sensitivity of 98.3% and a specificity of 93.8%.

Several studies reported a 100% accuracy. Finning KM et al. (2002) reported a 100 percent accuracy for the prediction of the fetal D-status from 137 D-negative women using the QIAmp Blood DNA Mini Kit in 2002. Hromadnikova I. et al. 2005 analyzed 45 pregnant women in the 11th to 40th weeks of pregnancy using the QIAmp Blood DNA Mini Kit and correlated the results with serological analysis of cord blood after delivery in 2005.

However, the amount of the fetal DNA is rather low, especially in early gestation. Geifman-Holtzman O et al. (2006) did a meta-analysis recently. They identified 37 English-written publications that included 44 protocols reporting non-invasive Rh genotyping using fetal DNA obtained from maternal blood. Their results showed that the diagnostic accuracy of noninvasive fetal Rh determination using maternal peripheral blood is 94.8%. So improvements of the technique may improve accuracy of testing and enable large-scale risk-free fetal RhD genotyping using maternal blood.

Since the concentration of fetal DNA in maternal blood is very low, unsatisfactory results occurred in prior experiments. The analysis of other fetal genetic traits is more problematic because of the overwhelming presence of the maternal DNA sequences in the circulation (Li Y. et al. 2004). In order to increase the detection sensitivity and accuracy, the fetal DNA should be extracted from the maternal plasma and enriched to higher concentration and purity (Hourfar M.K. et al. 2005). The method of DNA extraction is therefore very important and improvement in this technology is required.

In this thesis, two methods were developed to extract the fetal DNA from maternal plasma. Firstly, the QIAamp DSP Virus Kit was optimized to extract fetal DNA from maternal plasma. Secondly, a high-throughput method for fetal DNA separation from maternal plasma, which allows enrichment of small DNA fragments, was developed using specific fish-primer and hybridization capture.

4.1. Selecting the best material for optimization studies

In order to identify the best automated nucleic acid extraction method, it is not realistic and probably not ethically justified to take blood from a pregnant woman for such a purpose. If blood has been taken serially in order to control anti-D titer from one immunized woman carrying a D-

positive fetus, a pool of these samples is also of interest, however these cases are rare and these samples will probably have been stored for a long time. Those samples, which were used for RBC antibody testing, should be collected from D-negative pregnant women, as if these samples were taken for examination, informed consent from each pregnant woman will probably not be required provided that no personal data is collected. These details have been approved by the local ethical board.

Study material in this study was obtained from a co-operating laboratory where plasma from D-negative pregnant women was separated within a few days from cells from EDTA anti-coagulated blood samples. 1000 samples from D-negative women were pooled in order to get a mixture of samples with D-positive and D-negative fetuses. Since more than 50% of these women carry a D-positive child, the pooling will be close to a 1-in-2 dilution of material from a D-negative woman with a D-positive fetus.

4.2 Optimization of the QIAamp DSP Virus Kit for the extraction of fetal DNA from maternal plasma

Some research implied that carrier RNA could improve the efficiency of extraction of target RNA. The handbook of the QIAamp DSP Virus Kit also recommends using the carrier RNA to extract RNA. This purpose is to extract DNA. Although occasionally some papers implied that carrier RNA could enhance the recovery of RNA from dilute solutions (Kishore R 2006), it should be known whether the carrier RNA is beneficial for the extraction of DNA. Thus an experiment was designed in which one group of samples was tested with carrier RNA, and the other group of samples was tested without carrier RNA. There was no significant difference between the results of the 2 groups of experiments. Maybe the small amount of the samples in test (n=14 in our experiment) caused this result.

Using the protocol of the manufacturer it could be observed that it was difficult for the plasma to pass through the membrane in time. It was anticipated that the protein was not completely denatured and the pores of the membranes were blocked by the undenatured protein. The handbook of the method recommends using 75μl of protease. In order to study whether the amount of the protease could influence the passing time of the samples, the volume of protease was increased to 100μl, which reduced the time of vacuum separation. This change of the

protocol had no effect on the sensitivity of *RHD* PCR.

So far, the common viewpoint about the problem whether the DNA is eluted in water or AVE buffer is that DNA should be eluted in AVE buffer for preservation. When the DNA is not to be preserved, the DNA can be eluted in water. In one experiment, I found that the DNA eluted in water gave better data than DNA in AVE buffer. The reason for this result may be that the components in the AVE buffer could have some side effects on the PCR reactions.

In summary, the Ct value (mean ct 31.36 and 31.27) did not change when there was no carrier RNA in AL buffer (p=0.56, Fig.3-1). The DNA eluted in water (mean ct 32.17) is better than AVE buffer (mean ct 33.41, p<0.05, Fig.3-2). The percentage of denatured samples (n=24, each) that passed through the membrane within 15 min increased from 87.5% to 100% when 100 µl protease was used instead of 75µl. The 95% detection limit of the optimised protocol was 138 pg/ml (21 geq/ml, Fig.3-4).

The improved method of the QIAamp DSP Virus Kit can be used for larger prospective trials to detect fetal DNA from maternal plasma. It has a good efficiency and yield. The results showed that the optimised DSP had good performance in the experiments before. It could be concluded from Table 3-10 that the recovery of the optimised DSP method was much higher than other methods. In Fig.3-16, it could also be seen that the yield of the optimised manual DSP method was much higher than the yield of other manual methods including the QIAamp DNA Blood Mini Kit.

4.3 Fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

4.3.1 Number of fish primers

In almost all the experiments designed before by Legler TJ et al. (1999) and Heermann et al. (1996) they used only 1 fish primer. There is speculation that the >1-fish-primer design would be better because when more fish primers are used in the experiments, the sites of hybridization would increase compared with the 1-fish-primer design. In this study, the 2-fish-primer and 4-

fish-primer design was significantly better than the 1-fish-primer design, but there was no significant difference between the 2-fish-primer design and 4-fish-primer design. Since more fish primers cause higher costs, the 2-fish-primer design was selected.

4.3.2 Different location of the fish primers

It was found that the closer the fish primers were to the amplification primers, the lower were the values of Ct i.e. the higher the efficiency of the extraction of fetal DNA from the maternal plasma was.

The mechanism of the results can be explained as follows. In fact, wherever the fish primers locate, the quantity of the fetal DNA fragments will be the same statistically. But the closer the fish primers are to the amplification primers, the more target DNA fragments that match the amplification primers will be detected.

4.3.3 Optimization of the biotinylation of the fish primers

With fish primers biotinylated on 5' high Ct-values were observed even with plasma from D-negative blood donors. Therefore, it was tested whether fish primers alone are able to prime the PCR and may whether this may cause false positive reactions. When this could be demonstrated, 3`-5`-biotinylated primers were tested which gave more specific results. Since fish primers have to be longer than sequence specific amplification primers, this 3`-modification seems to be necessary in very sensitive real-time PCR.

4.3.4 Concentration of fish primers

Theoretically, the higher the concentration of the fish primers is, the more target DNAs will be extracted from the samples. However, the more fish primers, the more magnetic particles are required. The manufacturer of magnetic particles recommends that the concentration should not exceed 25 mg per PCR. Thus the concentration of fish primers was optimized. The experiments showed that 32nM fish primers (mean ct 35.7) were worse than 4nM (mean ct 34.4), 8nM (mean ct 34.5) and 16nM (mean ct 34.7) ($p<0.01$, Fig.3-7); 8nM fish primer were slightly better than

4nM and 16nM. (not significant).

4.3.5 The efficiency of extraction and enrichment of fetal DNA in HCMP

Since the concentration of fetal DNA in maternal blood is very low, many false positive results occurred in prior experiments. The analysis of other fetal genetic traits is more problematic because of the overwhelming presence of the maternal DNA sequences in the circulation (Li Y et al., 2004). In previous studies, viral RNA was specifically isolated by specific fish primers (Legler TJ et al., 1999). Therefore, this method was introduced for the isolation of fetal DNA. Specific fish primers were designed, which prefer to combine with the fetal DNA rather than maternal DNA. At the same time, some papers reported that the maternal-derived DNA molecules were longer than the fetal-derived ones, and the size of most of the fetal DNA fragments was less than 300bp (Chan KCA et al., 2004). In order to further increase the sensitivity, the condition of the hybridization was optimized, such as the selection of magnetic particles, the pH-value, etc. This optimization was beneficial for the binding of short fragments of DNA, fish primers and magnetic particles.

For the analysis of the percentage of fetal DNA in total DNA, specific primers and probes of *RHD* were designed that could detect fetal DNA, and specific primers and probes of *RHCE* that could detect total DNA. In order to evaluate the efficiency of the enrichment of fetal DNA using the HCMP method, a comparison with other methods is useful. Many DNA extraction methods were developed to extract genomic DNA from whole blood from smaller volumes (about 200 µl). For the separation of fetal DNA from plasma, larger volumes (500µl and more) are of interest because of the low concentration of fetal DNA in plasma. The comparison with the non-specific DNA extraction method of the DSP virus Kit implied that the method of HCMP could enrich the fetal DNA from circulation.

4.3.6 Summary

The two fish primer design is better than the one fish primer design in Ct values (p=0.014), but there is no difference between the design of the four fish primers and that of the two fish primers (p>0.05). The Ct values increased from 33.6 to 35.5(p<0.01) when fish primers were designed, located about 10bp and 200bp upstream or downstream from the amplification primers. 32 nM

fish primers (mean ct 35.7) were worse than 4nM(mean ct 34.4), 8nM(mean ct 34.5) and 16nM(mean ct 34.7); 8nM fish primers were slightly better than 4nM and 16nM. (Not significant). The optimal pH for hybridisation was 9.2 (mean ct 34.3). A pH of 8.6 (mean ct 35.5) and 9.8 (mean ct 35.2) gave higher ct values (p<0.02). The concentration of circulatory fetal DNA obtained with HCMP was increased by 150% compared with the QIAamp DSP Vacuum method. This difference was significant. (p=0.012). The 95% detection limit for HCMP of the extraction of fetal DNA from maternal plasma was determined at 358 pg/ml (54 geq/ml).

In conclusion, the method of hybridisation capture and magnetic particles can enrich circulatory fetal DNA. It has a good potential to purify fetal DNA from maternal DNA. However, in this part of the work, more than one fish primer was adapted. But not all the fish primer sequences were designed specifically. Thus the enrichment rate was still not very high. In future research, the specificity of fish primers has to be improved to increase the enrichment rate of fetal DNA.

4.4. High-throughput fetal DNA separation from maternal plasma by hybridization capture and magnetic particles

A manual HCMP was developed to extract and enrich the fetal DNA from total DNA. Using this approach, the amount and concentration of fish primers were optimized, as was the condition of hybridization and binding capture. The experiment results demonstrated that the efficiency and yield of my optimized method were higher than the current methods. However, since the details described in aim-2 were only for manual procedures, it is difficult to adapt them to routine tests. At the same time, the sensitivity and reproducibility of the method would be influenced. It is not easy to avoid cross-contamination. So in this part, this method will be automated. Meanwhile, the cost and time of the experiments would be considered as factors when evaluating the method. The purpose of my optimization is that this method can be used in routine clinical testing.

So far, to our knowledge, no papers have been published about the high-throughput extraction and enrichment of fetal DNA from maternal DNA. In the SAFE network (http://safenoe.org/) meeting, van der Schoot CE et al. reported a large-scale study applying a fully automated assay. She concluded that high-throughput non-invasive fetal *RhD* genotyping in the 30th week of pregnancy is at least as reliable as cord blood serology (more than 99% diagnostic accuracy). The assay costs for reagents and equipment are low, i.e. under € 15 per assay. However, her method was based on the MagnaPure (Roche) instrument, which is a non-specific method. Thus

the fetal DNA cannot be enriched. For early gestation (at the 8th to 12th week), it is difficult to detect the fetal DNA with satisfying sensitivity, because of the overwhelming presence of maternal DNA in the samples. If a method of fetal DNA enrichment could be developed, this problem may be resolved.

In 1996 Heermann KH et al. published a protocol for the extraction of HCV-RNA with specific capture of oligo-nucleotides and magnetic particles (Heermann KH et al., 1996). Their results implied that this method was beneficial in detecting the low concentration of HCV-RNA. In 1999, Legler TJ et al. modified this method to high-throughput and applied it as a screening method in a blood bank facility (Legler TJ et al., 1999). Since the concentration of fetal DNA in the maternal circulation is very low in the plasma, which is similar to the situation of HCV-RNA in the plasma, this method could be adapted for the extraction and testing of fetal DNA.

In this thesis, only a new method was provided. The samples used for testing were not from a single patient but were from pooled samples of many patients (n>30). Although a high yield can be achieved with this method of pooled samples, further research about the test of single samples should be performed.

In conclusion, the results of this part showed that it takes about 3.3h to test 96 samples using this method. The time required to test each sample was only about 2 mins. The efficiency and sensitivity of this method was higher than some other methods. Compared with some other commercial instruments this method has a higher facility and automation level. Cross-contamination could be avoided in the automated procedure. The 95% detection limit was also lower than the manual method.

4.5. Evaluation of the optimized DSP, hybridization capture and other methods for fetal DNA isolation from maternal plasma

During the development of the method of hybridization capture and magnetic particles, real-time PCR was based on *RHD* exon 7. Theoretically, these optimization results should be suitable for other genotyping methods. However, it is not enough to determine the *RhD* status only based on exon 7. The status of *RhDψ* should also be detected. Therefore, in the international survey, the status of *RhDψ* was also tested with real time PCR primers and probes for *RHD* exon 5.

This survey demonstrated substantive differences in the yield of cell-free fetal (cff) DNA between different DNA extraction methods when using maternal plasma samples. It is concluded that the optimized DSP Virus Kit might be the optimal candidate for a manual reference method. This conclusion is supported by the recent evaluation of the optimized DSP Virus Kit by an independent research group (Clausen FB et al, 2007). One likely reason for the lesser amount of cff DNA obtained by the QIAamp DNA Blood Mini Kit is that this is designed to extract mainly large fragments of genomic DNA, while cff DNA is known to be fragmented into <300bp segments (Chan KCA. et al., 2004; Li Y et al., 2004).

Comparable results were obtained with the QIAamp DNA Blood Mini Kit by different labs, although small differences lead to differences in sensitivity, possibly caused by omitting the centrifugation step of the empty column before elution to eliminate residual ethanol containing washing buffer. The QIAamp DNA Blood Midi Kit did not give satisfactory results in the lab, which used it in this survey. The CST genomic DNA purification Kit yielded results comparable with the QIAamp DNA Blood Mini Kit.

Compared with the automated methods, the BioRobot MDx (processing a 96 well QIAamp plate similar to the QIAamp MinElute spin columns used in the manual QIAamp DSP Virus Kit) showed the highest DNA recovery, but the starting volume was too low for plasma samples with low cff DNA concentration.

The results imply that the optimization DSP Virus Kit, the High Pure PCR Template Preparation Kit, an in-house protocol using the QIAamp DNA blood Mini Kit, the CST genomic DNA purification kit, the Magna Pure LC, the MDx, the M48 the EZ1 and manual HCMP for manual and automated extraction were the methods which were able to reliably detect fetal *RHD* in pools 1-3. The best results were obtained with the optimization DSP Virus Kit. The QIAamp DNA blood Mini Kit showed very comparable results in laboratories that followed the manufacturer's protocol and started with ≥500 µL plasma. One participant using the QIAamp DNA blood Midi Kit failed to reliably detect *RHD* in pool 3.

In conclusion, the optimized DSP Virus Kit is one of the best methods for extracting fetal DNA from maternal plasma. The manual and automated HCMP are comparable and potential methods for extracting fetal DNA from maternal plasma.

4.6. Technical details of the developed automated HCMP method and commercially available DNA extraction robots

In Table 4-1, the difference between some of the technical details of this method (Tecan/in house) and some other automated methods, such as MP LC (Roche, MagNa Pure LC), EZ1 (Qiagen), M48 (Qiagen) and MDx (Qiagen) is shown. In about 3.3h, 96 samples can be tested with this method. This efficiency is lower than MDx, but higher than MP LC, EZ1 and M48. In this method, the barcode can be read automatically. It also includes the functions of PCR setup and data export.

Table 4-1 shows the difference between some of the technical details of this method and some other automated methods

Methods	MP LC	EZ1	M48	MDx	Tecan/in-house
samples/run	32	6	48	96	96
Processing time	2 h	0.6 h	3.5 h	2.5 h	3.3 h
Barcode reader	hand BC	no	hand BC	integrated and hand BC	integrated BC
PCR setup	yes	no	yes	yes	yes
Data-Export	yes	no	yes	yes	yes
primary tubes	no	no	no	yes	yes

Appendix: The modified software of the optimization of automated liquid handling and pipetting steps
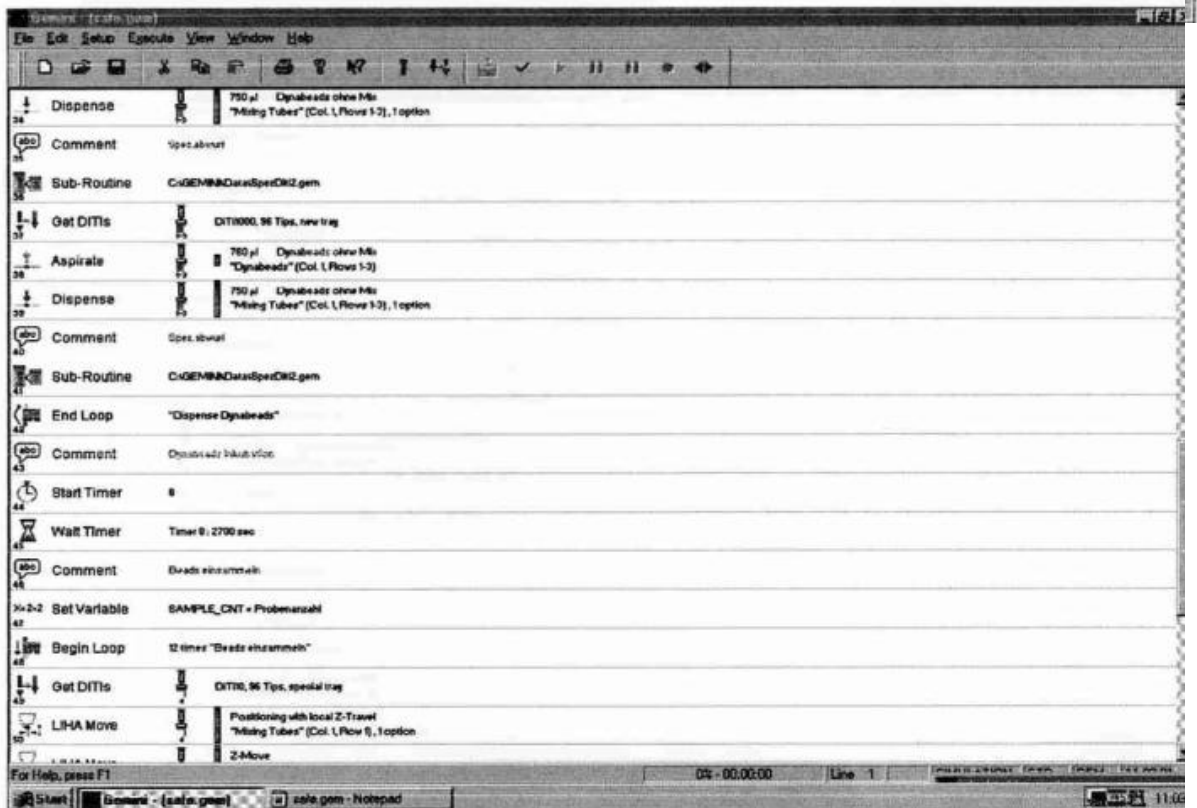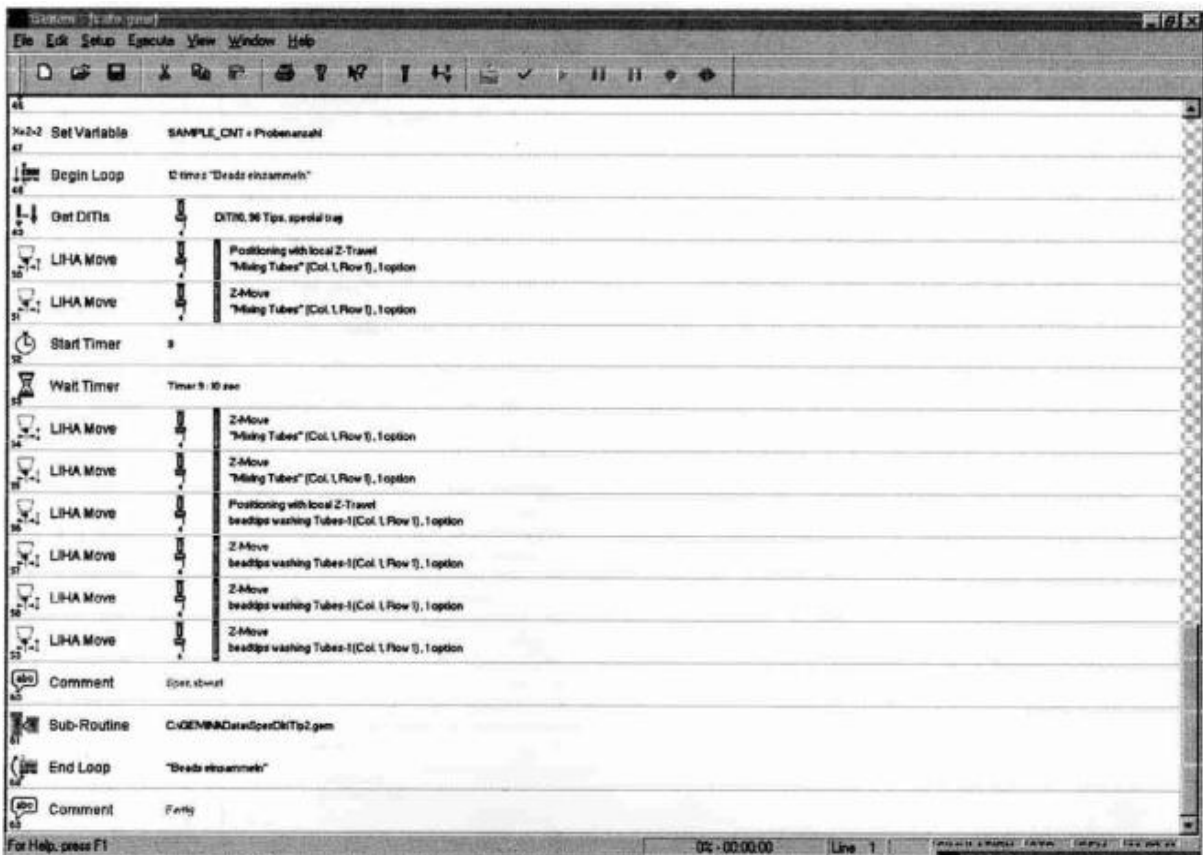
Icon	Command	Description
!?=	User Prompt	"Incubate the samples for 20 min at 60°C@Incubate at 95°C for 20 min@Hybridization at 60°C for 30 min" sound: once
!?=	User Prompt	"Are all Samles on board?" sound: once
X>Y?	Condition	1 = 1 Dynabeads mischen
↓	Wash Tips	8.0 - 0.1 ml
↓	Wash Tips	5.0 - 0.1 ml
abc	Comment	Dynabeads mischen
X>Y?	Condition	1 = 1 BEADS VERTEILEN
X=2+2	Set Variable	SAMPLE_CNT = 3
↓	Get DITIs	DITI1000, 96 Tips, new tray
Mix	Mix	3 x 990 µl Dynabeads ohne Mix "Dynabeads" (Col. 1, Rows 1-3)
↓	Drop DITIs	"Diti Waste"
abc	Comment	BEADS VERTEILEN
X=2+2	Set Variable	SAMPLE_CNT = Probenanzahl
↓	Begin Loop	16 times "Dispense Dynabeads"
↓	Get DITIs	DITI1000, 96 Tips, new tray
↑	Aspirate	760 µl Dynabeads ohne Mix "Dynabeads" (Col. 1, Rows 1-3)
↓	Dispense	760 µl Dynabeads ohne Mix "Mixing Tubes" (Col. 1, Rows 1-3), 1 option
abc	Comment	

66

Summary

To prevent anti-D immunization during pregnancy through the transplacental haemorrhaging of fetal erythrocytes in D-negative women, antenatal and postnatal anti-D prophylaxis is applied routinely in many countries, including Germany. However, anti-D is frequently not indicated. In a predominantly Caucasian population, 15-18 % of all pregnancies occur in D-negative women. In about 40% of all these cases the fetus is D-negative and antenatal anti-D prophylaxis is applied unnecessarily. Consequently, it would be beneficial if a method (especially a high-throughput method) was available for determining the fetal D type in all D-negative pregnant women.

Since Lo Y.M. reported that fetal DNA was present and could be detected in maternal plasma in 1997 (Lo YM. et al. 1997), many researchers have started to investigate the fetal RhD status from fetal DNA extracted from the maternal plasma. However, the amount of fetal DNA is rather low, especially early in gestation, and this kind of research is therefore facing a huge challenge.

In this thesis, two methods were developed for extracting fetal DNA from maternal plasma. Firstly, the QIAamp DSP Virus Kit (Qiagen) was optimized to extract fetal DNA from maternal plasma. The 95% detection limit was determined at 138 pg/ml (21 geq/ml). Secondly, a hybridization capture method combined with magnetic particles (HCMP) was developed using specific hybridization primers for the extraction of fetal DNA. The HCMP method was automated by adapting a commercially available liquid handling robot. The 95% detection limit was determined at 286 pg/ml (43 geg/ml).

In order to evaluate the two optimized methods, an international survey on the extraction of fetal DNA from maternal plasma was held. The highest yield was obtained by the optimized QIAamp DSP Virus extraction method, higher than the frequently used QIAamp DNA Blood Mini Kit. Among the automated methods the HCMP method showed results comparable with other instruments evaluated.

Key Words: Fetal DNA, RhD, noninvasive prenatal diagnosis, enrichment, high-throughput, hybridization capture, magnetic particles

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Abbreviation Items

A,C,G, T	Adenine, cytosin, guanidine, thymidine nucleotide
bp	Base pair
cff	Cell-free fetal
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
HDFN	Haemolytic Disease of the Fetus and Newborn
HEAD	High-throughput extraction, amplification, and detection
HCMP	Hybridization capture and magnetic particles
LT	Lysis Tube
NATs	Nucleic acid amplification techniques
NIPD	Non-Invasive Prenatal Diagnosis
QP	QIAGEN Protease
RAADP	Routine antenatal anti-D prophylaxis
PCR	Polymerase chain reaction
Rh	Rhesus
RhD	RhD protein
RHD	*RHD* gene
RhCE	RhCE protein
RHCE	*RHCE* gene
RT-PCR	Reverse transcription-PCR
SAFE NoE	The Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network of Excellence
VC	VacConnector
WT	Wash Tube
vCJD	Variant Creutzfeldt-Jakob disease

Acknowledgements

The dissertation benefited from the direction of several people.

Firstly, I owe my supervisor, Privatdozent Dr. med. Axel Pruß much appreciation. He always gave me many guidance and supports during every step of this research study including study design. Special gratitude is also expressed to Prof. Dr. med. Tobias J. Legler. During the time I spent at the University of Goettingen, whenever I encountered problems, he always gave me help. He gave me instructive comments at each step of my research works.

Secondly, I owe Prof. Dr. Dr. Holger Kiesewetter much appreciation for his guidance on my experimental and theoretical study. With his strong support I had the opportunity to do this research and finish my work for this thesis. I would like to thank him for his help. While I was studying at the university as an international student, he gave me much help and support.

Next, I would like to thank Privatdozent Dr. Klaus-Hinrich Heerman, and my colleagues in the research group and other friends. They also gave me much help not only in my research, but also in my life in Germany.

Finally, I would like to express special thanks to my wife, Ms. Lou Hong, my son, Liu Yifei and my parents, for their support and understanding.

Curriculum Vitae

LIU ZHONG

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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