The Glass Slide Extraction System Snap Card Improves Non-Invasive Prenatal Genotyping in Pregnancies with Antibodies

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Keywords

Cell-free DNA · Non-invasive prenatal genotyping · DNA extraction

Summary

Background: Determination of fetal blood groups in maternal plasma samples critically depends on adequate pre-analytical steps for optimal amplification of fetal DNA. We compared the extraction of cell-free DNA by binding on a glass surface (BCSI SNAP™ Card) with an automated system based on bead technology (MagnaPure compact™). **Methods:** Maternal blood samples from 281 pregnancies (7th–39th week of gestation) with known antibodies were evaluated in this study. Both the SNAP card and the MagnaPure method were applied to isolate DNA in order to directly compare the amplification in a single base extension assay and/or real-time PCR. **Results:** The mean concentration of total DNA obtained by the SNAP card (33.8 ng/μl) exceeded more than twofold that of MagnaPure extraction (15.7 ng/μl). SNAP card-extracted samples allowed to detect 3.7 single nucleotide polymorphisms (SNPs) versus 2.5 SNPs in MagnaPure extracts to control for traces of fetal DNA. This difference is highest for samples from 7th–13th week of gestation. **Conclusion:** The SNAP card system improves DNA extraction efficacy for prenatal diagnosis in maternal blood samples and provides an at least

eightfold higher total amount of DNA for the ensuing analysis. Its advantage is most evident for samples from early stages of pregnancy and thus especially valuable for pregnancies with antibodies.

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Introduction

Fetal red blood cell (RBC) antigens are relevant in the pathologic involvement of maternal alloantibodies causing hemolytic disease of the fetus (HDF) or newborn (HDN). For diagnostic procedures, traces of fetal DNA circulating in the plasma of pregnant women are nowadays used for determination of blood groups of the fetus [1]. This non-invasive approach introduces a valuable alternative to invasive procedures, e.g. amniocentesis or collection of fetal blood, provided that the validity of the non-invasive diagnosis is proven. The reliability of non-invasive *RHD* typing with real-time PCR was investigated in large studies during the past decade [2–13]. The majority of the studies focused on screening of *RHD*-negative pregnant women to assess the need for anti-D-prophylaxis during pregnancy and collected samples after the 20th week of gestation [14–20]. A valid result in fetal genotyping may, however, be clinically meaningful for the diagnostic monitoring of pregnancies as early as 7th–13th week of gestation, especially if antibodies are already detectable in the maternal serum. A negative result in fetal blood group genotyping from maternal blood samples remains inconclusive unless the presence of adequate amounts of

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Table 1. Investigated blood group systems and known antibodies / antibody titer of tested samples

Blood group	Samples	First antibody			Second antibody	
		specificity	samples	titer, range	specificity	titer, range
7th–13th week (n = 28)						
RHD	18	anti-D	16	1–32,768	anti-C anti-K	1–256 1024
KEL 1	8	anti-K	6	1–2,048		
RHc	1	anti-c	1	64		
RHE	1	anti-E	1	16		
14th–24th week (n = 208)						
RHD	136	anti-D	60	1–65,536	anti-C anti-E anti-Jk(a)	1–512 1–32 1–8
KEL 1	27	anti-K	21	1–8,192	anti-Jk(a)	1–64
RHE	18	anti-E	16	1–2,048	anti-c anti-Jk(a) anti-S	1–8 1–128 1–128
RHc	12	anti-c	12	1–512	anti-K anti-E anti-S	1–1,024 1–64 1–8
FY*A	6	anti-Fy(a)	2	1–512		
RHC	3	anti-C	3	1–32	anti-S	1
JK*A	2	anti-Jk(a)	2	1–8		
MNS 1	2	anti-M	1	2		
MNS 3	2	anti-S	2	1–64		
≥25th week (n = 45)						
RHD	22	anti-D	16	1–4,096	anti-C anti-E	1–64 1–8
RHE	10	anti-E	8	1–1,024	anti-c	1–64
KEL 1	3	anti-K	2	1–256		
RHc	5	anti-c	4	1–128	anti-K anti-S anti-Fy(a)	1–512 1–1,024 1
MNS 1	3	anti-M	3	1–8		
MNS 3	1	anti-S	1	1–32		
FY*A	1	anti-Fy(a)	1	1–512

Fig. 1. SNAP card system for binding of cfDNA to the glass surface.

fetal DNA is demonstrated for each individual sample. A valid control for cell-free fetal DNA (cffDNA), neither depending on the gender of the fetus nor requiring a paternal control sample, is essential to fully exploit the potential of methods. Deletion/insertion polymorphisms and single nucleotide polymorphisms (SNPs) are used to discriminate between fetal and maternal DNA on a qualita-

tive basis [21, 22]. Insufficient amplification of cffDNA could occur due to problems with the effectiveness of DNA extraction, especially if the cffDNA concentration in the maternal plasma is very low.

Thus, quality of a specific DNA extraction technology affects the analytical performance of the diagnostic method. Different

Table 2. Comparison of plasma-DNA extraction with MagnaPure and SNAP card technique

	All samples (n = 281)	Week of gestation		
		7th–13th (n = 28)	14th–24th (n = 208)	≥ 25th (n = 45)
<i>MagnaPure compact</i>				
DNA concentration, ng/μl (range)	15.7 (4.9–87.5)	13.6 (5.8–25.6)	15.8 (4.9–87.5)	17.0 (10.1–23.9)
Mean number of informative SNPs	2.6	1.7	2.8	2.1
Mean peak height (SBE)	1,253	998	1,269	1,308
qPCR (RHD only)				
Ct value	36.8 (n = 176)	37.3 (n = 18)	37.0 (n = 136)	35.5 (n = 22)
<i>SNAP card</i>				
DNA concentration, ng/μl (range)	33.8 (17.1–159.1)	32.9 (19.6–93.0)	33.5 (17.1–159.1)	36.3 (18.1–134.4)
Mean number of informative SNPs	3.7	3.3	3.9	2.9
Mean peak height (SBE)	1,596	1,750	1,633	1,455
qPCR (RHD only)				
Ct value	37.4 (n = 176)	38.2 (n = 18)	37.5 (n = 136)	36.6 (n = 22)
Mean fetal DNA yield (% of MagnaPure extraction)	116.2 (1.4–357.6)	143.5 (28.0–336.6)	117.7 (1.4–357.6)	87.6 (20.6–248.5)

technologies for extraction of maternal plasma, e.g. binding on spin columns or magnetic particles, have been evaluated for non-invasive prenatal blood group genotyping [23, 24].

DNA extraction from cell-free plasma by binding to the surface of a glass slide introduces an innovative approach for this purpose [25]. The SNAP card consists of an S-shaped plastic channel sandwiched by two glass slides. The sample can be flowed through the channel allowing contact between the sample and the flat glass surfaces to which the nucleic acids will bind (fig. 1).

We, therefore, compared this new method with our routine procedure based on magnetic particles to investigate plasma samples from 281 pregnant women with known antibodies or suspect of antibodies due to pregnancies in the past.

Material and Methods

Samples and DNA Preparation

EDTA-anticoagulated blood samples from 281 pregnant women (9th–36th week of gestation) were sent to our laboratory for routine non-invasive typing of fetal blood groups (table 1). Plasma was prepared according to Lo et al. [1]. DNA was extracted in parallel from 500 μl of plasma using the MagnaPure large volume DNA isolation kit (MagnaPure compact™, Roche Diagnostics, Grenzach-Whylen, Germany) and the BCSI SNAP card. (Blood Cell Storage Inc. Seattle, WA, USA). The binding time for cell-free DNA (cfDNA) on the glass surface of the SNAP card varied from 18–24 h followed by 2 × 3 automated wash steps with buffer I and II and 10 min air-drying of the card. The final elution volume was 200 μl (SNAP card) and 50 μl (MagnaPure), respectively.

Genomic DNA from maternal samples was isolated with the PureGeneD Kit according to the manufacturers' instructions (PureGene Blood Core Kit B; QIAgen, Hilden, Germany).

Quantification of the Total DNA Concentration

Total DNA yield and purity of the extracted DNA was examined by UV-spectroscopy (NanoDrop 1000; NanoDrop Technologies, Kisker, Steinfurt, Germany).

SNP Detection

DNA from 281 plasma samples (fetal DNA and maternal DNA for comparison) was screened for *RHD* exons 3, 4, 5 and 7 in a multiplex PCR setting in-

cluding 52 SNPs divided into 4 primer pools as described previously [21]. SBE products were identified due to dye and size with the GeneScan method in an ABI 310 (Applied Biosystems, Foster City, CA, USA) and analyzed with GenMapper™ software (version 4.0; Applied Biosystems) using the maximum-signal method for peak normalization.

Real-Time PCR

cfDNA from MagnaPure and SNAP card extraction was tested in duplicates for the presence of *RHD* exon 10 as reported in detail elsewhere [21]. For calculation of cfDNA, standard curves were included in each qPCR run. Determination of % fetal DNA yield was done similar to Clausen et al. [23]

Results

The comparison of both methods for DNA extraction was based on maternal samples from 281 pregnancies: 28 from a pregnancy at week 7–13 of gestation, 208 from week 14–24 of gestation, and 45 from week ≥ 25 of gestation. The concentration of total DNA in the eluates extracted in parallel from identical samples by both methods was measured. The quantitative PCR to detect *RHD* exon 10 was performed only in those samples (n = 176) known from their initial investigation to be *RHD*-positive. All maternal blood samples (n = 281) were typed for 52 SNPs with single base extension. The results are summarized in table 2.

DNA Extraction

The extraction of cfDNA by binding to a glass surface has been compared to our standard automated magnetic beads technique. The mean concentration of total DNA for all samples extracted with the SNAP card was 33.8 ng/μl (range 17.1–159.1 ng/μl) compared to 15.7 ng/μl (range 4.9–87.5 ng/μl) using magnetic particles. The substantially higher DNA extraction yield of SNAP card-extracted samples was confirmed for all samples, independent of the week of gestation. The extraction purity, characterized as ratio 260/280 nm, was 1.8 or higher for SNAP card preparation and varied between 1.3 and 1.5 for MagnaPure assay (data not shown).

Fig. 2. Single base extension assay for determination of paternal SNPs and real-time PCR for the detection of *RHD*-specific sequences (12th week of gestation). **A** Higher peaks of SNAP card DNA (937 rfu) compared to MagnaPure extraction (415 rfu). **B** *RHD*-positive results for SNAP card sample, MagnaPure and maternal sample were D-negative. **C** Positive internal control in SNAP card sample; no signal in MagnaPure extracted DNA. **D** Real-time PCR with positive results for SNAP card DNA.

Single Base Extension

Single base extension assays serving as gender-independent internal controls showed higher (fig. 2A) and/or more peaks for paternal SNPs in the SNAP card-extracted samples (fig. 2B,C). The number of informative SNPs calculated for all samples was 3.7 in cfDNA from SNAP card compared to 2.6 after MagnaPure extraction. Samples from week 7–13 s of gestation had a mean of 3.3 informative SNPs in extracts prepared by SNAP card versus 1.7 for extracts obtained by magnetic bead technology. The mean difference in peak height examined for all samples and 52 SNPs was 343 relative fluorescence units (rfu). The mean peak height difference for all samples collected in the 1st trimester was 752 rfu, decreasing to 364 for samples in the 2nd and 147 in the 3rd trimester.

Real-Time PCR

A difference of 0.5–1.1 was observed for threshold cycles (Ct values) in real-time amplification of *RHD* exon 10 comparing samples extracted with either method when a positive result was obtained. The mean yield of cfDNA (%) for all samples was 116.2 ($p < 0.0001$) with the highest value found in samples from the 1st

trimester (table 2). 14 MagnaPure-extracted samples (7th–13th week of gestation: $n = 3$; 14th–24th week of gestation: $n = 11$) failed in detection of *RHD* exon 10 while their SNAP card counterparts showed positive results.

Discussion

Non-invasive prenatal genotyping is an elegant approach to determine the fetal blood group genotype in pregnancies with known antibodies and at risk of HDN. The pre-analytical steps are essential for the success of the downstream applications, e.g. real-time PCR or fragment length analysis. In addition to the transport of the samples from the gynecologist to the laboratory [26] and the preparation of plasma, the extraction of cfDNA affects at least the sensitivity of the genotyping method [23, 24]. In addition, different extraction methods show different yields in cfDNA and cffDNA from plasma [23].

The SNAP card system bases on the capturing of DNA on untreated flat glass slides in the presence of chaotropic salts. The sys-

tem is automated with washing and elution steps, and only cell lyses and proteinase treatment of the sample have to be done manually, with a hand-on time of approximately 10 min. Special experiences of the operator are not necessary.

We designed a study to systematically evaluate this extraction technology for non-invasive prenatal genotyping. Maternal plasma samples stored at -80°C up to 96 months were introduced into this study and were compared to our standard DNA extraction system for prenatal genotyping [20]. These systems have been optimized to detect the presence of both the *RHD* gene and sufficient amounts of cfDNA.

The results demonstrate the advantages of the extraction of cfDNA from plasma using glass slides. The SNAP card system provides both a higher concentration as well as a higher absolute yield of total nucleic acids. In respect to the different elution volume of the methods, the absolute yield of DNA was fourfold higher with the SNAP card procedure. A mean amount of $6.76\text{ }\mu\text{g}$ total DNA was isolated by the SNAP card system in comparison to $0.79\text{ }\mu\text{g}$ total DNA extracted by the MagnaPure procedure. The quality of the total nucleic acids, indicated by a 260/280 nm ratio of ≥ 1.8 , was better in SNAP card technique. Interestingly, the higher ratio did not affect the results of the real-time PCR. In contrast, the Ct values of MagnaPure-extracted samples were lower than the SNAP card counterparts (0.5–1.1). Since both samples were tested in the identical real-time PCR run, the Ct values may be the result of a lower qPCR efficiency because of different composition of elution buffers of both extraction methods. Nevertheless, the mean yield of cfDNA (%) was significantly higher in samples extracted with the SNAP card, especially in samples of the 1st trimester.

Both the number and the height of single base extension peaks were higher in DNA samples prepared with the SNAP card

method. Calculated for all samples, the number of single base extension peaks, serving as internal controls for cfDNA, was 3.7 (SNAP card) and 2.6 (MagnaPure). Interestingly, samples from the 7th–13th week of gestation provided 3.3 informative SNPs when using glass-bound DNA compared to 1.7 for magnetic bead-bound DNA. In addition, the internal controls in this subgroup showed higher values for SNAP card DNA (MagnaPure 998 rfu; SNAP card 1,750 rfu).

An example is given in figure 2 for a pregnancy at 12th week of gestation and an initial anti-D antibody of 2,048 rfu. In contrast to the MagnaPure extraction, the SNAP card DNA provided valid *RHD* typing results based on successful real-time PCR and single base extension assays. Such a failure of the typing with our standard extraction method in contrast to the SNAP card system was observed in 3/18 samples from the 1st trimester.

In our study, 94% of the samples collected from gestational week 7–13 were from women with at least one antibody. Our results emphasize the benefit of the glass slide extraction method in early pregnancies.

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Disclosure Statement

The authors declare that they have no conflicts of interest relevant to this manuscript.

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