Introduction of Noninvasive Prenatal Testing for Blood Group and Platelet Antigens from Cell-Free Plasma DNA Using Digital PCR

Marion Eryilmaz · Dennis Müller · Gabi Rink · Harald Klüter · Peter Bugert

Institute of Transfusion Medicine and Immunology, Heidelberg University, Medical Faculty Mannheim,
German Red Cross Blood Service Baden-Württemberg – Hessen, Mannheim, Germany

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Abstract

Background: Noninvasive prenatal testing (NIPT) for fetal antigens is a common standard for targeted immune prophylaxis in RhD-mediated hemolytic disease of the fetus and newborn, and is most frequently done by quantitative PCR (qPCR). A similar approach is considered for other blood group and human platelet alloantigens (HPA). Because of a higher sensitivity compared to qPCR for rare molecule detection, we established and validated digital PCR (dPCR) assays for the detection of *RHD* exons 3, 5 and 7, *KEL1*, *HPA-1a*, and *HPA-5b* from cell-free DNA (cfDNA) in plasma. The dPCR assays for the Y-chromosomal marker *amelogenin* and autosomal SNPs were implemented as controls for the proof of fetal DNA. **Methods:** Validation was performed on dilution series of mixed plasma samples from volunteer donors with known genotypes. After preamplification of the target loci, two-color (FAM and VIC) TaqMan™ probe chemistry and chip-based dPCR were applied. The assays for *RHD* included *GAPDH* as an internal control. For the diallelic markers *KEL1/2*, *HPA-1a/b*, *HPA-5a/b*, and *AMEL-X/Y* and 3 autosomal SNPs, the probes enabled allelic discrimination in the two fluorescence channels. The dPCR protocol for NIPT was applied to plasma samples from pregnant women. **Results:** The *RHD*

exon 5 assay allowed the detection of a 0.05% *RHD* target in an RhD-negative background, whereas the exon 7 assay required at least a 0.25% target. The exon 3 assay showed the highest background and required at least a 2.5% *RHD* target for reliable detection. The dPCR assays for the diallelic markers revealed similar sensitivity and enabled the detection of at least a 0.5% target allele. The *HPA-1a* assay was the most sensitive and allowed target detection in plasma mixtures containing only 0.05% *HPA-1a*. The plasma samples from 13 pregnant women at different gestational ages showed unambiguous positive and negative results for the analyzed targets. **Conclusion:** Analysis of cfDNA from maternal plasma using dPCR is suitable for the detection of fetal alleles. Because of the high sensitivity of the assays, the NIPT protocol for RhD, *KEL1*, and *HPA* can also be applied to earlier stages of pregnancy.

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Introduction

The immunization in pregnancy against a blood group or platelet antigen of the fetus can cause severe complications such a hemolytic disease of the fetus and newborn (HDFN) or fetal and neonatal alloimmune thrombocytopenia (FNAIT). The development of maternal alloantibodies against the RhD antigen can be circumvented by the administration of immunoglobulins. In Germany and

other countries, anti-D prophylaxis is given to all D-negative pregnant women irrespective of the fetal D antigen status. Based on allele frequency, it can be estimated that about 35% of D-negative pregnant women do not require anti-D prophylaxis because the fetus is D negative [1]. The use cell-free DNA (cfDNA) from maternal plasma to identify the RhD status of the unborn child is well known [2]. This noninvasive prenatal testing (NIPT) for RhD is established as nationwide screening in different European countries [3–5]. The standard procedure includes blood sampling in gestational week 24–27 and quantitative real-time PCR (qPCR) targeting two *RHD* exons, i.e., exons 5 + 7 or 5 + 10 or 7 + 10. A high sensitivity (0.01–0.09% false-negative results) and specificity (0.1–0.9% false-positive results) of qPCR could be demonstrated. Other blood group antigens such as Rhc, RhE, and Kell (KEL1) can also cause HDFN [6]. Different NIPT methods have been developed based on qPCR or next-generation sequencing; however, none of them is implemented in a consecutive screening program [7–9].

The human platelet alloantigens (HPA) 1a and 5b are the most common cause of FNAIT. Similar to the antibody-mediated destruction of red blood cells in HDFN, maternal alloimmunization against HPA-1a or HPA-5b leads to immune thrombocytopenia in the fetus. In contrast to the RhD genetics, i.e., lack of the entire *RHD* gene in RhD-negative individuals, a single nucleotide polymorphism (SNP) in the corresponding glycoprotein gene represents the molecular basis of the HPA-1 and -5 antigens [10]. With regard to sensitivity and specificity, the detection of SNP alleles is much more challenging than the detection of *RHD* exons. Different NIPT methods using qPCR and next-generation sequencing have been developed, but consecutive screening of HPA-1a- and HPA-5b-negative pregnant women is not established yet [11–13]. The introduction of digital PCR (dPCR) offered significant advantages in the specific and sensitive detection of genetic markers. Compared to qPCR, a better signal-to-noise ratio results in a higher sensitivity of dPCR. Additionally, absolute quantification is possible with dPCR, without the use of standard curves. A dPCR method for the detection of *RHD* exons 5, 7, and 10 in cfDNA showed results comparable to those obtained with qPCR [14]. However, for rare molecule detection of SNP alleles or point mutations, dPCR is superior to qPCR [15]. dPCR reliably detected 0.1% rare alleles, whereas qPCR required at least 1% rare alleles for stable detection.

The focus of this work is the establishment and technical validation of chip-based dPCR assays for NIPT of RhD, KEL1, HPA-1a, and HPA-5b from cfDNA. Our main intention was to allow the detection of cell-free fetal DNA (cffDNA) in early pregnancy, especially in cases with maternal alloantibodies. With regard to anti-D prophylaxis, the dPCR methods can also be used for screen-

ing in the second trimester. The sensitivity of the assays was evaluated using mixed plasma samples of donors with known genotypes. In addition, we introduced control assays for detection of the Y-specific allele of the gonosomal target amelogenin (AMEL) and for autosomal SNPs from the SNPforID panel [16, 17]. All assays were based on two-color (FAM and VIC) TaqMan™ technology with previously published primers and probes [14, 18, 19] or commercial assays that have been used before in a genotyping project [20]. Finally, the validated dPCR assays were exemplarily applied to plasma samples of pregnant women and confirmed the high sensitivity and specificity for the detection of the rare (fetal) alleles.

Materials and Methods

Preparation of Plasma Samples and Isolation of cfDNA

The validation study was performed on blood samples from healthy volunteer donors (staff of the institute) after they had given their consent. The validated dPCR protocol was applied to plasma samples from pregnant women who also gave informed written consent. The study protocol was approved by the local ethics committee (Vote No. 2017-665N-MA). The blood samples were stored and shipped at room temperature and processed within 3 days after sampling. Plasma was obtained from EDTA blood after centrifugation for 10 min at 2,000 g, transferred to new centrifuge tubes, and centrifuged at 16,000 g for 10 min at 4 °C. Cell-free plasma was then transferred to cryotubes and stored at –30 °C until use.

For the sensitivity tests, we prepared dilution series (1 mL final volume each) of mixed plasma samples with 5%, 1%, 0.5%, and 0.1% plasma of a donor heterozygous for the target (*RHD*, KEL1, HPA-1a, HPA-5b, and AMEL-Y) and 95%, 99%, 99.5%, and 99.9% plasma of a donor negative for the target, respectively. All tests were conducted with biological triplicates from different donors. Isolation of DNA from 1 mL cell-free plasma was conducted with the QIAmp Circulating Nucleic Acids Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cfDNA was eluted from the column using 100 µL sterile water, and the DNA concentration was measured photometrically (NanoDrop Lite; Thermo Fisher Scientific GmbH, Dreieich, Germany). From a total of 72 isolations, the range of cfDNA concentration was 2.1–19.5 ng/µL (5.0 ± 2.3).

Preamplification PCR

In order to increase the number of evaluable signals in dPCR, all loci were preamplified by PCR using primers flanking the dPCR primers (Table 1). The preamplification PCR was performed in 25 µL multiplex reactions with Multiplex PCR Master Mix (Qiagen), 0.1 µmol of each primer, and 2–9 ng cfDNA. The cycling program included 15 min at 95 °C, 30 cycles with 30 s at 94 °C, 90 s at 63 °C, 90 s at 72 °C, followed by 10 min at 72 °C. Multiplex I included primers for *RHD* exons 3, 5 and 7, *GAPDH* and *AMEL*, multiplex II included primers for *KEL*, HPA-1, HPA-5 and *AMEL*, and multiplex III included primers for control SNP1, SNP2 and SNP3. After PCR, the products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was measured by NanoDrop Lite (Thermo Fisher Scientific).

Table 1. Primers for the preamplification PCR and TaqMan™ assays for dPCR

Target	Primer sequence for preamplification (5'–3') and amplicon size	Primer and probe sequence (5'–3') or commercial assay code (FAM or VIC label as indicated)
<i>RHD</i> exon 3	for: TCTCAGTCGTCCTGGCTCTC rev: TTAAGTATGACCATCCTCAGGT amplicon size: 175 bp	for: GGCCACCATGAGTGCTTTG rev: CTCCACCAGCACCATCACC P: FAM-TGCTGATCTCAGTGGATG-MGB
<i>RHD</i> exon 5	for: TTCTGGCCAACCACCCTCTC rev: GTCACCACGCTGACTGCTAC amplicon size: 148 bp	for: CGCCCTCTTCTTGTGGATG rev: GAACACGGCATTCTTCCTTTC P: FAM-TCTGGCCAAGTTTCAAC-MGB
<i>RHD</i> exon 7	for: CAGCTCCATCATGGGCTACAA rev: GGGTAAGCCCAGTGACCC amplicon size: 122 bp	for: TGTGCTGCTGGTGCTTGA rev: AGTGACCCACATGCCATTG P: FAM-ACCGTCGGAGCCG-MGB
<i>GAPDH</i>	for: CCATCCCTTCTCCCCACAC rev: GCTGTATTTTAACCCCTAGTCC amplicon size: 123 bp	for: CCCCACACACATGCACTTACC rev: CCTAGTCCCAGGGCTTTGATT P: VIC-TAGGAAGGACAGGCAAC-MGB
<i>AMEL</i>	for: CTGGGCACCCTGGTTATATC rev: CTTGAGGCCAACCATCAGAG amplicon size: 201 bp	for: CCCTGGGCTCTGTAAAGAATAGTG rev: ATCAGAGCTTAACTGGGAAGCTG Px: VIC-TATCCCAGATGTTTCTC-MGB Py: FAM-CCAAATAAAGTGGTTTCTC-MGB
KEL1/KEL2 (rs8176058)	for: GGCTCCACGGATCCTTATG rev: TGTGTGGAGAGGCAGGATG amplicon size: 178 bp	C_1719_20 KEL1: FAM; KEL2: VIC
HPA-1a/b (rs5918)	for: TGGACTTCTCTTTGGGCTCC rev: TTGAGTGACCTGGGAGCTG amplicon size: 189 bp	C_818008_30 HPA-1a: FAM; HPA-1b: VIC
HPA-5a/b (rs1801106)	for: ACACCATTACAGACGTGCTC rev: CTTTCCAAATGCAAGTTAAATTACC amplicon size: 165 bp	C_27862812_10 HPA-5a: FAM; HPA-5b: VIC
SNP1 (rs1357617)	for: CTCATTGGCAGCTGATGCAG rev: GTCTCAAACGCCATCAGTATAG amplicon size: 169 bp	C_11354314_10 rs1357617-T: FAM; rs1357617-A: VIC
SNP2 (rs2830795)	for: GGCTGCAGGTTGATGATTTTC rev: TCTACCCAGATGCCTGAAATATG amplicon size: 189 bp	C_3086569_20 rs2830795-G: FAM; rs2830795-A: VIC
SNP3 (rs1028528)	for: AACAGATGTCCACAGCTGATG rev: AGCAAGGGCATGGGGATCCA amplicon size: 177 bp	C_2988456_20 rs1028528-G: FAM; rs1028528-A: VIC
dPCR, digital PCR; MGB, minor groove-binding quencher.		

The concentration and the mean size of the preamplification products represented the basis for calculating the number of DNA molecules for dPCR by the following formula:

$$\frac{\text{DNA molecules}/\mu\text{L}}{(\text{ng}/\mu\text{L preamplification product}) \times \text{Avogadro constant}} = \frac{1}{(\text{mean bp size of PCR products}) \times 660 \times 10^9}$$

Avogadro constant = 6.022×10^{23} molecules/mol; 660 g/mol = average mass of 1 bp double-stranded DNA; 10^9 = factor for g to ng.

In order to elucidate the optimal number of DNA molecules, we performed pilot experiments ($n = 3$) in which we subjected 2×10^7 , 4×10^6 , 8×10^5 , 1.6×10^5 , 3.2×10^4 , and 6.4×10^3 DNA mol-

ecules per chip to dPCR analysis of *RHD* exon 7 in 5% RHD mixed plasma samples.

Assay Design and Chip-Based dPCR

dPCR (QuantStudio™ 3D; Thermo Fisher Scientific) was performed on chips with 20,000 reaction wells, each with a 755-pL volume. We used self-designed (*RHD*, *GAPDH*, and *AMEL*) and commercial (*KEL*, HPA-1, HPA-5, and control SNPs) assays based on the TaqMan™ technology with two fluorescent dyes, FAM and VIC (Table 1). The assay design was based on previously published primer and probe sequences for *RHD* exons 3, 5 and 7, *GAPDH*, and *AMEL* [14, 18, 19]. The assays for *RHD* were designed with FAM-labeled probes specific for exon 3, 5, or 7, and each included

Fig. 1. Representative scatter plots of the *RHD* exon 7 digital PCR assay. Yellow dots: no amplification (ROX signal); red dots: positive reactions for *GAPDH* (VIC signal); blue dots: positive reactions for *RHD* exon 7 (FAM signal); green dots: positive reactions for both targets (VIC + FAM signal). The Poisson-corrected values of target copies/ μ L and % target/total are given. Upper panel: result from a plasma mixture with 1% D-positive plasma from an *RHD/d* heterozygous donor, i.e., the proportion of the *RHD* allele was 0.5%. Lower panel: results from a D-negative plasma sample demonstrating the background of the assay.

GAPDH as a reference target detected by a VIC-labeled probe. The *AMEL* assay contained a Y-specific probe (FAM channel) and an X-specific probe (VIC channel) as an internal control. All other assays detected diallelic SNPs with one allele in the FAM channel (KEL1, HPA-1a, HPA-5b, SNP1a, SNP2a, and SNP3a) and one allele in the VIC channel (KEL2, HPA-1b, HPA-5a, SNP1b, SNP2b, and SNP3b). The *KEL*, HPA-1, and HPA-5 assays have been used before in a genotyping study [20].

The self-designed assays for *RHD* exons 3, 5, and 7 were prepared as 40 $\times$ concentrated mixtures containing 18 μ mol of each *RHD* primer, 3 μ mol of each *GAPDH* primer, and 5 μ mol of each probe. The 40 $\times$ *AMEL* assay contained 18 μ mol of each *AMEL* primer and 5 μ mol of each *AMEL* probe. The commercial assays were purchased as 40 $\times$ concentrates (Thermo Fisher Scientific). For each dPCR analysis, 4 $\times$ 10⁶ preamplified DNA molecules in 7.1 μ L were mixed with 0.375 μ L 40 $\times$ TaqManTM assay and 7.5 μ L dPCR Master Mix V2 containing ROX as a reference dye (Thermo Fisher Scientific). The reaction mixture was loaded onto a chip and covered with oil using the QuantStudioTM 3D Chip Loader device (Thermo Fisher Scientific). The cycling program for dPCR of *RHD* exon 7, *AMEL*, *KEL*, SNP1, SNP2, and SNP3 consisted of 10 min at 96 $^{\circ}$ C followed by 40 cycles with 30 s at 98 $^{\circ}$ C and 2 min at 56 $^{\circ}$ C. For *RHD* exon 3, *RHD* exon 5, HPA-1, and HPA-5, the annealing temperature was 52 $^{\circ}$ C.

Analysis of dPCR Results

After cycling, the dPCR chips were scanned for the FAM and VIC signals (QuantStudioTM 3D Chip Reader; Thermo Fisher

Scientific) and the data were uploaded into the QuantStudioTM 3D AnalysisSuite cloud software (<https://apps.thermofisher.com/quantstudio3d>). Based on the fluorescence signals and statistical correction using Poisson distribution, the software allowed the calculation of target copies per microliter and target/total (%) values. In addition, the uncorrected raw data were displayed in scatter plots (Fig. 1). Poisson correction is necessary to consider reaction wells containing more than one target molecule [21].

The QuantStudioTM 3D AnalysisSuite cloud software provided the final results; however, to give a deeper understanding of how the values are calculated, the procedure is briefly explained. First, the numbers of filled wells (ROX positive) and wells negative for FAM or VIC were determined and used to calculate P_0 for the FAM channel and the VIC channel:

$$P_0 = \frac{\text{Number of FAM- or VIC-negative wells}}{\text{Number of filled wells}}.$$

Second, the correction factor λ was introduced to consider the probability of more than one target molecule in a filled well: $\lambda = -\ln P_0$.

Third, the concentration c (copies/ μ L) was calculated:

$$c = \frac{\lambda}{V};$$

the reaction volume (V) of a single well on the chip is 755 pL = 7.55 $\times$ 10⁻⁴ μ L.

Table 2. Results from digital PCR analysis of *RHD* exon 7 in a dilution series of preamplified cell-free DNA from 5% mixed plasma samples

Preamplified molecules/chip	Target copies/ μL^a	Target/total ^a , %
2×10^7	295.5 $\pm$ 208.3	5.14 $\pm$ 2.13
4×10^6	10.6 $\pm$ 7.8	4.86 $\pm$ 0.87
8×10^5	4.5 $\pm$ 3.5	5.82 $\pm$ 1.79
1.6×10^5	2.1 $\pm$ 1.2	4.44 $\pm$ 0.90
3.2×10^4	0.5 $\pm$ 0.2	10.51 $\pm$ 4.29
6.4×10^3	0.4 $\pm$ 0.0	30.65 $\pm$ 4.40

^a Mean $\pm$ SD values from biological triplicates.

Table 3. Results of the *RHD*, *KEL*, and *HPA* digital PCR assays from the mixed plasma samples

Target	% heterozygous plasma (% target allele)	Target copies/ μL^a	Target/total ^a , %
<i>RHD</i> exon 3	5.0 (2.5)	33.6 $\pm$ 13.6	3.54 $\pm$ 0.36
	1.0 (0.5)	4.1 $\pm$ 0.9	1.00 $\pm$ 0.44
	0.5 (0.25)	5.2 $\pm$ 2.9	0.49 $\pm$ 0.12
	0.1 (0.05)	1.5 $\pm$ 0.4	0.38 $\pm$ 0.19
	0.0 (0)	4.9 $\pm$ 3.1	0.60 $\pm$ 0.20
<i>RHD</i> exon 5	5.0 (2.5)	18.0 $\pm$ 4.9	6.47 $\pm$ 2.04
	1.0 (0.5)	6.3 $\pm$ 4.4	2.06 $\pm$ 0.72
	0.5 (0.25)	2.0 $\pm$ 1.1	0.85 $\pm$ 0.77
	0.1 (0.05)	2.3 $\pm$ 3.1	0.28 $\pm$ 0.33
	0.0 (0)	0.0 $\pm$ 0.0	0.00 $\pm$ 0.01
<i>RHD</i> exon 7	5.0 (2.5)	82.6 $\pm$ 44.8	4.36 $\pm$ 0.88
	1.0 (0.5)	36.9 $\pm$ 25.3	2.20 $\pm$ 0.45
	0.5 (0.25)	2.5 $\pm$ 3.2	0.48 $\pm$ 0.65
	0.1 (0.05)	0.7 $\pm$ 0.3	0.08 $\pm$ 0.03
	0.0 (0)	0.6 $\pm$ 0.7	0.04 $\pm$ 0.05
<i>KEL</i> 1	5.0 (2.5)	33.5 $\pm$ 1.5	2.33 $\pm$ 0.27
	1.0 (0.5)	8.8 $\pm$ 5.3	0.64 $\pm$ 0.47
	0.5 (0.25)	5.4 $\pm$ 5.0	0.40 $\pm$ 0.40
	0.1 (0.05)	1.6 $\pm$ 0.4	0.08 $\pm$ 0.01
	0.0 (0)	1.6 $\pm$ 0.5	0.07 $\pm$ 0.02
<i>HPA</i> -1a	5.0 (2.5)	83.5 $\pm$ 36.0	2.32 $\pm$ 1.01
	1.0 (0.5)	22.1 $\pm$ 21.2	0.63 $\pm$ 0.73
	0.5 (0.25)	8.0 $\pm$ 4.8	0.71 $\pm$ 0.37
	0.1 (0.05)	1.4 $\pm$ 0.1	0.12 $\pm$ 0.03
	0.0 (0)	0.1 $\pm$ 0.0	0.01 $\pm$ 0.01
<i>HPA</i> -1b	5.0 (2.5)	46.1 $\pm$ 4.2	2.41 $\pm$ 0.36
	1.0 (0.5)	17.0 $\pm$ 5.9	0.76 $\pm$ 0.31
	0.5 (0.25)	9.5 $\pm$ 4.5	0.58 $\pm$ 0.25
	0.1 (0.05)	6.7 $\pm$ 2.5	0.31 $\pm$ 0.03
	0.0 (0)	4.4 $\pm$ 0.2	0.28 $\pm$ 0.11
<i>HPA</i> -5b	5.0 (2.5)	25.8 $\pm$ 4.7	1.91 $\pm$ 0.47
	1.0 (0.5)	20.3 $\pm$ 23.1	0.55 $\pm$ 0.28
	0.5 (0.25)	1.8 $\pm$ 1.1	0.08 $\pm$ 0.00
	0.1 (0.05)	2.6 $\pm$ 1.6	0.13 $\pm$ 0.06
	0.0 (0)	2.1 $\pm$ 1.4	0.07 $\pm$ 0.01

^a Mean $\pm$ SD values from biological triplicates.

These calculations were carried out for both channels FAM and VIC. With the concentration of molecules (c_{FAM} and c_{VIC}), the target/total ratio was calculated

– for target detection in the FAM channel:

$$\text{target/total (\%)} = \frac{c_{\text{FAM}}}{c_{\text{FAM}} + c_{\text{VIC}}} \times 100;$$

– for target detection in the VIC channel:

$$\text{target/total (\%)} = \frac{c_{\text{VIC}}}{c_{\text{FAM}} + c_{\text{VIC}}} \times 100.$$

Results

Preamplification of the Target Loci

The standard protocol for dPCR is designed for the analysis of nanograms of genomic DNA; however, the amount of cfDNA in plasma samples is much lower. In preliminary experiments, we used cfDNA directly in dPCR for genotyping *RHD* or SNPs, and, as expected, the number of evaluable signals was rather low (<1,000; not shown). In order to increase the number of evaluable signals, we introduced preamplification of the target loci. The optimal number of preamplified target molecules for dPCR was established in mixed plasma samples containing 5% heterozygous D-positive plasma and 95% D-negative plasma.

cfDNA was extracted from the plasma mixtures and conducted to preamplification PCR with multiplex I, containing primers for *RHD*, *GAPDH*, and *AMEL*. After purification, dilution series from 2×10^7 to 6.4×10^3 molecules per chip of the preamplification products were analyzed by dPCR for *RHD* exon 7 (Table 2). The analysis of 2×10^7 , 4×10^6 , 8×10^5 , and 1.6×10^5 molecules revealed similar results of approximately 5% target/total; however, an overload of the chips was observed for 2×10^7 molecules. Lower numbers of molecules (3.2×10^4 and 6.4×10^3) lead to overestimation of the target/total ratio. The results obtained from 4×10^6 molecules were the most robust; thus, 4×10^6 preamplified molecules were used in all dPCR experiments described below.

Validation of the dPCR Assays

Plasma samples from volunteer donors with known genotypes for the investigated targets were used to validate the dPCR assays with regard to sensitivity and specificity. In order to simulate a fetal DNA fraction in the plasma of pregnant women, we mixed plasma of donors negative for the target allele with plasma of heterozygous donors in biological triplicates, i.e., 3 different donors each. All tests were performed on plasma mixtures containing 5%, 1%, 0.5%, and 0.1% heterozygous plasma. In addition, the background of the assays was tested in plasma samples of donors negative for the target. For testing *RHD*, specific assays for the detection of exons 3, 5, and 7

Fig. 2. Representative scatter plots of the HPA-1 digital PCR assay. Yellow dots: no amplification (ROX signal); red dots: positive reactions for the HPA-1b allele (VIC signal); blue dots: positive reactions for the HPA-1a allele (FAM signal); green dots: positive reactions for both alleles (VIC + FAM signal). The Poisson-corrected values of target copies/ μ L and % target/total are given. Upper panel: result from a plasma mixture with 1% HPA-1ab heterozygous plasma, i.e., the proportion of the HPA-1a allele was 0.5%. Lower panel: results from an HPA-1bb plasma sample demonstrating the background of the assay.

were established. *GAPDH* was used as a reference target in all *RHD* assays to allow target/total calculation. *RHD* exons 5 and 7 were detected reliably in 5%, 1%, and 0.5% of the mixed plasma samples containing 2.5%, 0.5%, and 0.25% of the target, respectively (Table 3; Fig. 1). The background of the exon 5 and 7 assays in D-negative plasma revealed $0.00 \pm 0.01\%$ and $0.04 \pm 0.05\%$ target/total, respectively. The higher background for the exon 3 assay ($0.60 \pm 0.20\%$ target/total), prevented specific *RHD* detection in the 1.0%, 0.5%, and 0.1% plasma mixes (Table 3). Technical triplicates of dPCR from a 5% mixed plasma sample indicated a coefficient of variation of 14% and 5% for the *RHD* exon 5 and exon 7 assays, respectively.

The Kell (KEL1) blood group antigen is encoded by the variant allele (T) of the diallelic SNP c.578C>T in the *KEL* gene. The validation of the assay for detection of the *KEL1* allele revealed a background of $0.07 \pm 0.02\%$ and a high sensitivity to detect the target in 1% and 5% mixed plasma samples (Table 3). With regard to FNAIT, the HPA-1a antigen is the clinically most relevant one and is encoded by the wild-type allele (T) of the diallelic SNP c.176T>C in the *ITGB3* gene. The high sensitivity and specificity of the HPA-1 assay allowed a reliable detection of the HPA-

1a allele even in the 0.1% mixed plasma samples (0.05% target) with $0.12 \pm 0.03\%$ target/total (Table 3; Fig. 2). The background was very low at $0.01 \pm 0.01\%$ target/total. The HPA-1 assay was also validated for detection of the HPA-1b allele, which revealed a significantly higher background of $0.28 \pm 0.11\%$ target/total. Therefore, the detection limit for the HPA-1b allele was higher than for the HPA-1a allele and required at least 0.5% mixed plasma (Table 3). For the HPA-5 assay, we found a lower sensitivity and a higher background than for the HPA-1 assay. Reliable detection of the clinically relevant HPA-5b allele could be achieved in 5% and 1% mixed plasma. The background values and the values for the 0.1% and 0.5% mixed plasma were very similar (Table 3).

The gonosomal locus *AMEL* contains a dimorphism with a 6-bp insertion on the Y chromosome compared to the X chromosome [16]. We established a dPCR assay for *AMEL* as a control marker for male fetus detection. The Y-chromosomal allele could be detected reliably in 5% and 1% mixed plasma. The background was $0.14 \pm 0.05\%$ target/total and overlapped with the values for 0.5% and 0.1% mixed plasma (Table 4). In order to introduce additional control markers for the detection of fetal DNA,

we selected diallelic SNPs from the SNPforID panel [17]. In this proof of principle, we tested three SNP markers for both alleles and used 1% plasma mixtures and also determined the background for the assays. All assays allowed reliable detection of the target alleles in the 1% plasma mixtures and showed a low background in the target-negative plasma (Table 4).

Analysis of Maternal Plasma

In a pilot study, the dPCR methods for the targets (*RHD* exons 5 and 7, *KEL1*, *HPA-1a*, and *HPA-5b*) and the control markers (*AMEL-Y* and *SNP1-3*) were applied to plasma samples from 13 selected cases of pregnancy at different gestational ages (Table 5). In 10 cases, the mothers were D negative, and the cfDNA analysis for *RHD* exons 5 and 7 indicated a D-positive fetus in 9 cases. Case No. 6 was negative for both *RHD* exons. The *KEL1* allele was analyzed in 12 cases, and all were negative. The *HPA-1a* allele could be analyzed in 3 *HPA-1bb* mothers, and 1 case (No. 2) showed a positive result in plasma cfDNA. Nine of the mothers were *HPA-5aa*, and the *HPA-5b* allele was detectable in case No. 3. The Y-chromosomal marker *AMEL-Y* was analyzed in all samples and indicated a male fetus in 3 cases, including the RhD-negative case No. 6. In case No. 4, a positive control for cfDNA was missing because all of the analyzed targets (*KEL1*, *HPA-1a*, and *HPA-5b*) and control markers (*AMEL-Y* and *SNP1-3*) were negative. Further markers need to be analyzed in this case to prove the presence of reasonable amounts of fetal DNA in the maternal plasma. A DNA sample of the newborn was available in 3 of the 13 cases, and the proposed fetal genotypes from NIPT could be confirmed.

Discussion

We presented the validation of NIPT assays for blood cell antigens using plasma cfDNA and chip-based dPCR. Pre-amplification of the target loci was introduced in order to increase the sensitivity of the assays as a prerequisite for the detection of fetal alleles at an early stage of pregnancy. Further improvement of sensitivity may be achieved when using specialized blood collection tubes (Cell-Free DNA BCT® CE; Streck Inc., La Vista, NE, USA) instead of EDTA tubes to prevent genomic DNA release from nucleated blood cells. It has to be shown whether the sensitivity of dPCR assays with cfDNA from Streck tubes without pre-amplification is comparable to our protocol with EDTA tubes and pre-amplification of the targets. Even without pre-amplification, a higher sensitivity of dPCR compared to qPCR for *RHD* exon 5 and 7 detection in samples with <2% cfDNA was demonstrated [22]. However, we suppose that pre-amplification

Table 4. Results of the control assays from the mixed plasma samples

Target	% heterozygous plasma (% target allele)	Target copies/μL ^a	Target/total ^a , %
AMEL-Y	5.0 (2.5)	6.4±2.2	4.20±1.70
	1.0 (0.5)	4.8±6.2	1.34±1.01
	0.5 (0.25)	0.4±0.2	0.23±0.18
	0.1 (0.05)	0.2±0.1	0.23±0.20
	0.0 (0)	0.2±0.1	0.14±0.05
SNP1a (rs1357617A)	1.0 (0.5)	3.5±1.2	0.52±0.16
	0.0 (0)	0.5±0.4	0.01±0.01
SNP1b (rs1357617T)	1.0 (0.5)	16.2±9.2	1.96±0.73
	0.0 (0)	0.0±0.0	0.00±0.00
SNP2a (rs2830795G)	1.0 (0.5)	27.0±7.7	3.24±0.38
	0.0 (0)	0.4±0.3	0.01±0.00
SNP2b (rs2830795A)	1.0 (0.5)	8.9±4.3	1.52±0.80
	0.0 (0)	0.1±0.0	0.00±0.00
SNP3a (rs1028528G)	1.0 (0.5)	6.0±5.3	0.24±0.01
	0.0 (0)	0.5±0.1	0.01±0.01
SNP3b (rs1028528A)	1.0 (0.5)	18.1±11.6	0.56±0.07
	0.0 (0)	1.0±0.5	0.03±0.01

^a Mean ± SD values from biological triplicates.

is advantageous to the sensitive detection of fetal SNP alleles.

Our technical validation supported the use of dPCR for NIPT in early stages of pregnancy. Detection of *RHD* exons 5 and 7 in an RhD-negative background was feasible in plasma mixtures containing only 0.25% *RHD* allele. In 3 cases of RhD-negative mothers, *RHD* exon 7 could be detected at an early stage (gestational weeks 9 and 12). However, the detection of SNP alleles such as *KEL1*, *HPA-1a*, *HPA-5b*, and others is more challenging regarding sensitivity and specificity. Our NIPT assays allowed SNP allele detection in plasma mixtures containing at least 0.5% of the targeted allele. In the validation study, the *HPA-1a* assay was the most sensitive and allowed the detection of even a 0.05% target. A similar sensitivity was reported for rare SNP allele detection using digital droplet PCR [15]. In 1 case of an *HPA-1bb* mother, we were able to detect the fetal *HPA-1a* allele at gestational week 13.

Our dPCR assays allow the detection of rare molecules in cfDNA and are, therefore, suitable for NIPT. For the detection of fetal *RHD*, *HPA*, and other blood group alleles using a massive parallel sequencing method, a 2% target/total cutoff was defined for reliable detection of a target allele [12]. We found that the background (false-positive signals) in dPCR is significantly lower and the target/total cutoff can in general be defined as 0.5%. In

Table 5. Results given in target copies/ μ L (% targets/total) from digital PCR analysis of cell-free DNA from pregnant women

Case No.	GA, week	<i>RHD</i> exon 5	<i>RHD</i> exon 7	KEL1	HPA-1a	HPA-5b	AMEL-Y	SNP1	SNP2	SNP3	Proposed fetal genotypes
1	25	20.1 (0.37)	56.3 (0.96)	0.3 (0.01)	0.9 (0.01)	0.3 (0.01)	0.4 (0.02)	nt	nt	b: 10.6 (0.25)	Dd; KEL2; HPA-1bb; HPA-5aa; XX; SNP3ab
2	13	nt	nt	0.1 (0.00)	8.0 (0.18)	0.3 (0.01)	0.8 (0.01)	nt	nt	nt	KEL2; HPA-1ab; HPA-5aa; XX
3	26	nt	nt	0.0 (0.00)	nt	2.6 (0.23)	3.1 (0.06)	b: 2.1 (0.05)	nt	nt	KEL2; HPA-5ab; XX; SNP1aa
4	9	nt	nt	0.1 (0.00)	1.6 (0.02)	1.2 (0.02)	0.7 (0.01)	a: 1.8 (0.02)	b: 1.0 (0.04)	a: 0.1 (0.00)	KEL2; HPA-1bb; HPA-5aa; XX; SNP1bb; SNP2aa; SNP3bb
5	39	1,104.3 (14.00)	989.2 (33.97)	0.2 (0.00)	nt	nt	0.5 (0.01)	nt	b: 1.4 (0.03)	nt	Dd; KEL2; XX; SNP2aa
6	34	0.9 (0.10)	0.4 (0.01)	0.1 (0.00)	nt	0.6 (0.01)	46.9 (0.82)	nt	b: 1.9 (0.04)	nt	dd; KEL2; HPA-5aa; XY; SNP2aa
7	36	221.8 (3.04)	709.2 (7.14)	0.2 (0.00)	nt	nt	8.0 (0.15)	nt	b: 0.8 (0.03)	b: 0.5 (0.01)	Dd; KEL2; XY; SNP2aa; SNP3aa
8	33	20.2 (0.43)	408.8 (3.82)	0.1 (0.00)	nt	1.5 (0.03)	0.4 (0.01)	a: 2.2 (0.08)	nt	nt	Dd; KEL2; HPA-5aa; XX; SNP1bb
9	12	nt	269.9 (12.30)	nt	nt	2.1 (0.06)	0.2 (0.13)	b: 81.5 (12.94)	b: 20.0 (3.01)	nt	Dd; KEL2; HPA-5aa; XX; SNP1ab; SNP2ab
10	9	16.1 (0.44)	18.0 (0.56)	1.3 (0.04)	nt	nt	0.8 (0.05)	nt	b: 6.8 (0.31)	b: 7.8 (0.27)	Dd; KEL2; XX; SNP2ab; SNP3ab
11	9	40.4 (1.24)	79.8 (3.51)	0.0 (0.00)	nt	0.5 (0.10)	9.4 (1.23)	a: 2.8 (0.10)	b: 10.8 (0.45)	nt	Dd; KEL2; HPA-5aa; XY; SNP1bb; SNP2ab
12	24	54.2 (1.71)	196.4 (4.99)	0.1 (0.00)	nt	1.7 (0.02)	0.2 (0.01)	b: 0.3 (0.01)	nt	nt	Dd; KEL2; HPA-5aa; SNP1aa
13	21	nt	129.5 (5.82)	0.0 (0.00)	nt	nt	0.6 (0.16)	nt	nt	nt	Dd; KEL2; XX

The targeted SNP allele (a or b) is indicated; positive results are highlighted (gray shading); a DNA sample of the newborn was available in cases No. 3, 5, and 11. GA, gestational age; nt, not tested because maternal genomic DNA was positive, or the SNP was heterozygous.

our hands, chip-based dPCR is a robust method; during validation, dPCR had failed and had to be repeated in only about 1% of the tests.

The combination of antenatal and postnatal administration of anti-D immune prophylaxis is a current standard to prevent HDFN in D-negative women [23]. Since the availability of anti-D immunoglobulin is limited, targeted use for antenatal prophylaxis is a reasonable contribution to health economics [24, 25]. The immune destruction of blood cells (in HDFN or FNAIT) mediated by other antigens can be prevented by antenatal administration of high-dose intravenous immunoglobulin [26]. Antigen-specific immune prophylaxis (anti-KEL1 or others) should have fewer side effects, but it is not available.

To prevent immune destruction of platelets in FNAIT, the EU-funded PROFNAIT consortium is developing an immune prophylaxis against HPA-1a-mediated FNAIT (www.profnait.eu).

Prenatal determination of the fetal antigen status is a prerequisite for establishing targeted immune prophylaxis. In countries with nationwide RhD-NIPT, gestational week 25–27 is the common time point for the screening of D-negative women. In most protocols, *RHD* exons 5 and 7 are detected by qPCR methods. Our dPCR protocol is based on the same TaqMan assays [14, 18] and includes preamplification of the target loci. However, preamplification is supposedly not needed for *RHD* detection at later gestational ages. In the pilot study, *RHD* exon 7 detec-

tion revealed high numbers of target copies per microliter in samples from the second or third trimester of pregnancy. In these cases, dPCR is probably not advantageous with regard to sensitivity and specificity when compared to qPCR. The availability of an internal control is a benefit of dPCR, whereas the costs per target (approx. EUR 10) are significantly higher than for qPCR.

RHD genotyping is hampered by the highly homologous *RHCE* gene. Rearrangement of the two genes lead to *RHD-RHCE* hybrid genes with exons from one gene replaced by the corresponding exons of the other gene. This is a common molecular basis of partial RhD phenotypes and has to be considered in *RHD*-specific exon amplification by PCR methods [27, 28]. The most common partial D phenotype is category DVI, which is caused by replacement of *RHD* exon 5 and others. Our and other NIPT for the detection of *RHD* exon 5 would be negative. However, the detection of exon 7 would be positive and the anti-D prophylaxis is appropriate. The molecular basis of the partial D phenotype DBT is the *RHD-RHCE* hybrid gene with rearranged exons 5–7 or 5–9 [29, 30]. NIPT results would be negative, but the DBT phenotype is characterized by the presence of D epitopes that can immunize a D-negative mother. In such a case, the anti-D prophylaxis would be appropriate but would not be administered, because of the negative NIPT result for exons 5 and 7. However, DBT alleles (*RHD*14*) are rare in Caucasian and other populations. The NIPT analysis of a third exon, i.e., *RHD* exon 3 or exon 10, could further increase the safety of detection of *RHD* variants, and assays for qPCR have been published [14, 19].

In addition to Y-chromosomal markers, anonymous autosomal SNPs have been considered and investigated as control markers to prove the presence of fetal cfDNA in maternal plasma [19]. Recent progress in this field has mainly been based on massive parallel sequencing in order to detect paternally inherited SNP alleles in maternal plasma [31, 32]. We established and validated dPCR assays for three autosomal SNPs from the SNPforID panel [17] to demonstrate the feasibility of sensitive SNP typing from cfDNA. In 1% plasma mixtures, the targeted SNP alleles could be reliably detected. The assays showed very low backgrounds in the absence of the target allele. Our panel of dPCR assays will be extended to additional autosomal SNPs.

In summary, we were able to show the suitability of dPCR for NIPT of blood group and platelet antigens from cfDNA. For validation of dPCR, we predominantly used mixed plasma samples from volunteer donors and only a limited number of plasma samples from pregnant women. A clinical validation study has been initiated to determine the sensitivity and specificity of the assays, which is necessary before implementing NIPT in routine care. The risk of false-positive results or contamination has to be

considered for this PCR method, especially because of preamplification. Therefore, in our standard protocol, we implemented the processing of control plasma (negative for RHD, KEL1, HPA-5b, and Y-chromosome) in parallel to the test samples. The work flow for routine diagnosis includes determination of the maternal genotypes for the requested targets and for the control SNPs from genomic DNA. Plasma cfDNA is then analyzed for the requested targets and the homozygous control SNPs using dPCR. Including plasma separation, cfDNA isolation, preamplification, and dPCR analysis, up to 5 test samples in addition to the control plasma can be analyzed each for 4 targets (e.g., *RHD* exon 5, *RHD* exon 7, and 2 control SNPs; total: 6 samples on 24 chips) within a working day. We established a standardized and robust procedure that can be easily transferred to further NIPT targets. Automation of the chip-based dPCR is desirable, but this is not possible with the current devices. Irrespective of the method used, the result of NIPT is uncertain if no target or control is positive. In such cases, the number of controls should be increased to prove the presence of reasonable amounts of cfDNA.

Statement of Ethics

This validation study was performed on blood samples from healthy volunteer donors (staff of the institute) after they had given their consent. The validated dPCR protocol was applied to plasma samples from pregnant women who also gave informed written consent. The study protocol was approved by the local ethics committee (Vote No. 2017-665N-MA).

Disclosure Statement

The authors declare no conflict of interest.

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