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Real-world performance of a clinical droplet digital polymerase chain reaction assay for non-invasive foetal blood group and platelet antigen genotyping of alloimmunized pregnant women with antibodies directed against RhD, RhE, Rhc, RhC, K1, HPA-1a or HPA-5b: A 1-year experience

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Real-world performance of a clinical droplet digital polymerase chain reaction assay for non-invasive foetal blood group and platelet antigen genotyping of alloimmunized pregnant women with antibodies directed against RhD, RhE, Rhc, RhC, K1, HPA-1a or HPA-5b: A 1-year experience

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Abstract

Background and Objectives: To test the performance of a new droplet digital polymerase chain reaction (ddPCR) non-invasive foetal blood group and platelet antigen genotyping assay in the setting of a Dutch reference laboratory for foetal blood group and platelet antigen genotyping. Our population comprised 229 consecutive alloimmunized pregnant women who presented between April 2022 and March 2023 with 250 requests for non-invasive foetal *RHD*, *RHE*, *RHc*, *RHC*, *K1*, *HPA-1a* or *HPA-5b* blood group and platelet antigen genotyping.

Materials and Methods: Samples were genotyped for blood group and platelet antigen alleles along with methylated *RASSF1a* (*mRASSF1a*) and sex-determining region of Y (*SRY*) and *DYS14* as positive foetal controls. Negative blood group and platelet antigen results were issued only when foetal controls were positive; otherwise, such samples were classified as inconclusive.

Results: The assay achieved a success rate of 98.4% (246 of 250) because one case was lost to follow-up, one case was solved with quantitative polymerase chain reaction (qPCR) and one case precluded foetal typing due to *RHD* variant mothers. Only

10 cases needed a second sample and one case a third for a valid final result. We identified 116 maternal–foetal blood group and platelet antigen incompatibilities.

Conclusion: Clinical non-invasive foetal blood group and platelet antigen typing of alloimmunized pregnant women via ddPCR is successful and represents an improvement over qPCR because of the addition of a foetal control and because ddPCR circumvents potential interference from maternal cell-free DNA (cfDNA) background for foetal HPA-1 and K1.

Keywords

alloimmunization, ddPCR, FNAIT, HDFN, pregnancy

Highlights

- We report our clinical experience with a new seven-target foetal blood group and platelet antigen genotyping assay.
- The new genotyping assay uses integrated foetal and maternal DNA controls.
- The assay was found to have a high level of performance, requiring only minimum repeat testing and repeat sample requests.

INTRODUCTION

Maternal–foetal blood group and platelet antigen incompatibility can cause maternal alloimmunization against foetal red blood cells [1] or foetal platelets [2]. This can cause haemolytic disease of the foetus and newborn (HDFN) or foetal and neonatal alloimmune thrombocytopenia (FNAIT), with possible severe consequences for the foetus's health [1, 3]. With timely detection, HDFN and FNAIT can be successfully treated through appropriate monitoring and adequate medical care [4]. Therefore maternal– foetal blood group and platelet antigen incompatibility should be determined in the early second trimester.

In Northern and Western European countries, non-invasive foetal blood group and platelet antigen genotyping is commonly performed using cell-free foetal DNA (cffDNA) extracted from maternal plasma using real-time quantitative polymerase chain reaction (RT-qPCR) technology [5–9]. However, for foetal genotyping at an early gestational age (GA), RT-qPCR presents several limitations, including potential background signal due to non-specific amplification of maternal genetic material, which was further reduced for K-genotyping by a new peptide nucleic acid (PNA) probe design [7], or enzymatic digestion of the *HPA-1b* allele [8]. One of the main disadvantages of RT-qPCR is that it is difficult to implement a control for the presence of foetal DNA [10,11,12] and reviewed in Reference [13]. The generic foetal identifier methylated *RASSF1a* (*mRASSF1a*) can show low levels of amplification in control samples from non-pregnant women, and is therefore less reliable at early GA when foetal DNA concentration might still be extremely low. For that reason, a set of multiplex RT-qPCRs with in/del polymorphisms has been developed that can reliably be used to show the presence of foetal DNA [10,13]. However, not in all maternal–foetal combinations can paternal markers be identified, and this approach is highly labour-intensive. Droplet digital polymerase chain reaction (ddPCR) represents a methodological

innovation of RT-qPCR, where, thanks to an emulsion of oil and water, thousands of independent polymerase chain reactions (PCRs) reactions take place simultaneously [14]. The partitioning of the PCR allows for a reduced background signal derived from maternal genetic material while maintaining high sensitivity. This technology therefore allows the implementation of the universal foetal marker *mRASSF1a*, which confirms presence of foetal DNA independently of the foetus's gender [10,15,16,17]. Other laboratories have already shown the feasibility of applying ddPCR technology for foetal blood group and platelet antigen typing [18,19]. At Sanquin Diagnostic Services, we have performed non-invasive foetal genotyping for more than 10 years. Recently, we chose ddPCR technology to improve the assay by including foetal markers in cffDNA extracted from blood of alloimmunized pregnant women identified by clinical serology with anti-RhD, RhC, Rhc, RhE or K and anti-platelet antigens HPA-1a and HPA-5b. The purpose of this study is to report the real-world performance of this ddPCR foetal genotyping test in a single institution over a 1-year period.

MATERIALS AND METHODS

Sample collection and cfDNA extraction

Samples were collected from 229 consecutive, pregnant, alloimmunized cases presenting to our lab between April 2022 and March 2023. Previously validated EDTA or Streck tube (Streck Corporation, Omaha, NE, USA) was used for maternal blood collection (30 mL whole blood). Blood was collected at different GAs and processed upon receipt or maximally after 48 h of collection. Blood samples were centrifuged at 1200×g for 10 min, and the resulting supernatant plasma was re-centrifuged in a new tube at 2400×g for 20 min. The

supernatant plasma was then transferred to new tubes and stored at -20°C until further processing. Cell-free DNA (cfDNA) was then extracted per patient from duplicate 3-mL plasma aliquots (A and B) for the Quick-cfDNA Serum & Plasma kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. In summary, the cfDNA from 30-mL whole blood was eluted in 60 μL volume.

Droplet digital polymerase chain reaction

ddPCR was performed using the QX200 AutoDG Droplet Generator and the QX200 ddPCR reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). For each extraction, technical duplicates ($2 \times 9 \mu\text{L}$ cfDNA eluate) were performed for alleles of sex-determining region of Y (SRY)/DYS14, ($2 \times 9 \mu\text{L}$ cfDNA eluate) *RHD*, *RHc*, *RHC*, *RHE*, *K*, *HPA-1a* or *HPA-5b*, and triplicates ($3 \times 5 \mu\text{L}$ cfDNA eluate) for *mRASSF1a*. For cases with two (or more) alloantibodies, each case was tested in a separate ddPCRs in a similar way as if independent cases were tested. In contrast to the *mRASSF1a*–*ACTB* multiplex ddPCR, eluates for blood group and platelet antigen PCRs were heat-denatured before droplet generation to double the number of target-containing droplets.

Real-time qPCR

Real-time qPCR was performed with the StepOnePlus RT-PCR system (Applied Biosystems). Primers and probes used in RT-qPCR for *RHD* detection are the same as used for ddPCR [8,20].

RASSF1a was amplified in multiplex with *ACTB*, and 0.5 μL HhaI and Bsh1236I methylation-sensitive restriction nucleases (Thermo Fisher) were included in the PCR mix. PCR used target-specific forward and reverse primers and probes (Table S1) and ddPCR SuperMix for probes (no dUTP) (BioRad 186-3024). The blood group and platelet antigen genotyping multiplex PCR was performed by combining 9 μL cfDNA eluate, target-specific forward, reverse primers and probe and ddPCR SuperMix for probes. Droplets were then generated with QX200 AutoDG, and samples proceeded to incubation in a C1000 Touch thermal cycler with the following incubation conditions: 37°C for 60 min (for the restriction of *mRASSF1a*), 95°C for 10 min, 40 cycles of 94°C for 30 s, 57°C for 1 min, 10 min at 98°C , 30 min at 4°C and 12°C on hold.

To control for cfDNA input, for each ddPCR, simultaneously the amount of total genomic DNA was measured (e.g., *ACTB*). In each assay, non-template controls (NTCs) served as negative controls and blood group and platelet antigen pos gDNA as positive controls. Primers and probe sequences used for the test are listed in Table S2. After PCR amplification, droplets were read using the QX200 droplet reader, and the results were analysed using the BioRad QuantaSoft software. The ddPCR results were then interpreted (Method M1 in Supporting Information) to reach a conclusion (foetal blood group and platelet antigen positive, negative or inconclusive) and generate a clinical report. The thresholds for positive detection were *RHD* (exons

5 and 7 combined) ≥ 4 droplets per well and ≥ 2 droplets per well for *K*, *RHc*, *RHC*, *RHE*, *HPA1a*, *HPA5b*, *mRASSF1a* and *SRY* in quadrant 1 (ch1+ch2–). For *DYS14*, the threshold was 33 copies per mL plasma. An inconclusive result is issued if one or more Quality Control (QC) gates fail (Method M1 in Supporting Information), if blood group or platelet antigen results in technical duplicates are discordant or if a maternal variant precludes a foetal genotype result.

Sanger sequencing and multiple-ligation-dependent probe amplification (MRC Holland Cat EK5-FAM, P401-100R, P402-100R, P403-100R) were performed according to standard laboratory methods.

Statistical methods

All calculations of percentage, mean, median and correlation were performed using Microsoft® Excel® for Microsoft 365 MSO (version 2205 Build 16.0.15225.20028) 32-bit, while the unpaired, two-sided t-test was performed on a Jupyter Notebook using SciPy 1.9 in Python 3.9.7 (default, 16 September 2021, 16:59:28) MSC v.1916 64 bit (AMD64).

RESULTS

Results of foetal blood group and platelet antigen genotyping via ddPCR

Between April 2022 and March 2023, Sanquin Diagnostic Services received and processed a total of 250 foetal blood group and platelet antigen test requests from 229 alloimmunized pregnant women (Figures 1 and 2; Tables 1 and S2). Most requested tests were for the assay predicting the *RhE* (*RH3*) phenotype ($n = 91$, with 38 *RHE* pos, 52 *RHE* neg, one lost to follow-up but tested *RHE* negative in qPCR to substitute for ddPCR), followed by *RhD* (*RH1*) ($n = 59$, with 31 *RHD* pos and 26 neg, two maternal variants), *K* (*K1*) ($n = 41$, with 17 *K1* pos and 24 neg), *Rhc* (*RH4*) ($n = 31$, with 14 *RHc* pos and 16 neg and 1 lost to follow-up) and *RhC* (*RH2*) ($n = 16$ with 6 *RHC* pos and 10 neg). Twelve cases presented with platelet alloimmunity and were tested for *HPA-1a* ($n = 4$, with three *HPA-1a* pos, one neg) and *HPA-5b* ($n = 8$, with seven *HPA-5b* pos and one neg) (Table S2). In 20 pregnancies, the foetuses were tested for more than one target of interest because the mothers presented with more than one alloantibody (Table S2, column case ID numbers highlighted).

Gestational age

The GA of the 229 cases of first blood samples was 18.6 weeks ($n = 217$; Interquartile range (IQR) = 7 [14–21 weeks], $n = 12$ unknown) and of second blood samples 20.8 weeks ($n = 10$; IQR = 6 [18–24 weeks], $n = 1$ unknown) and a third sample (Case 211) was collected at GA24. The GA distribution for all tests performed is given per target of interest in Figure 1b.

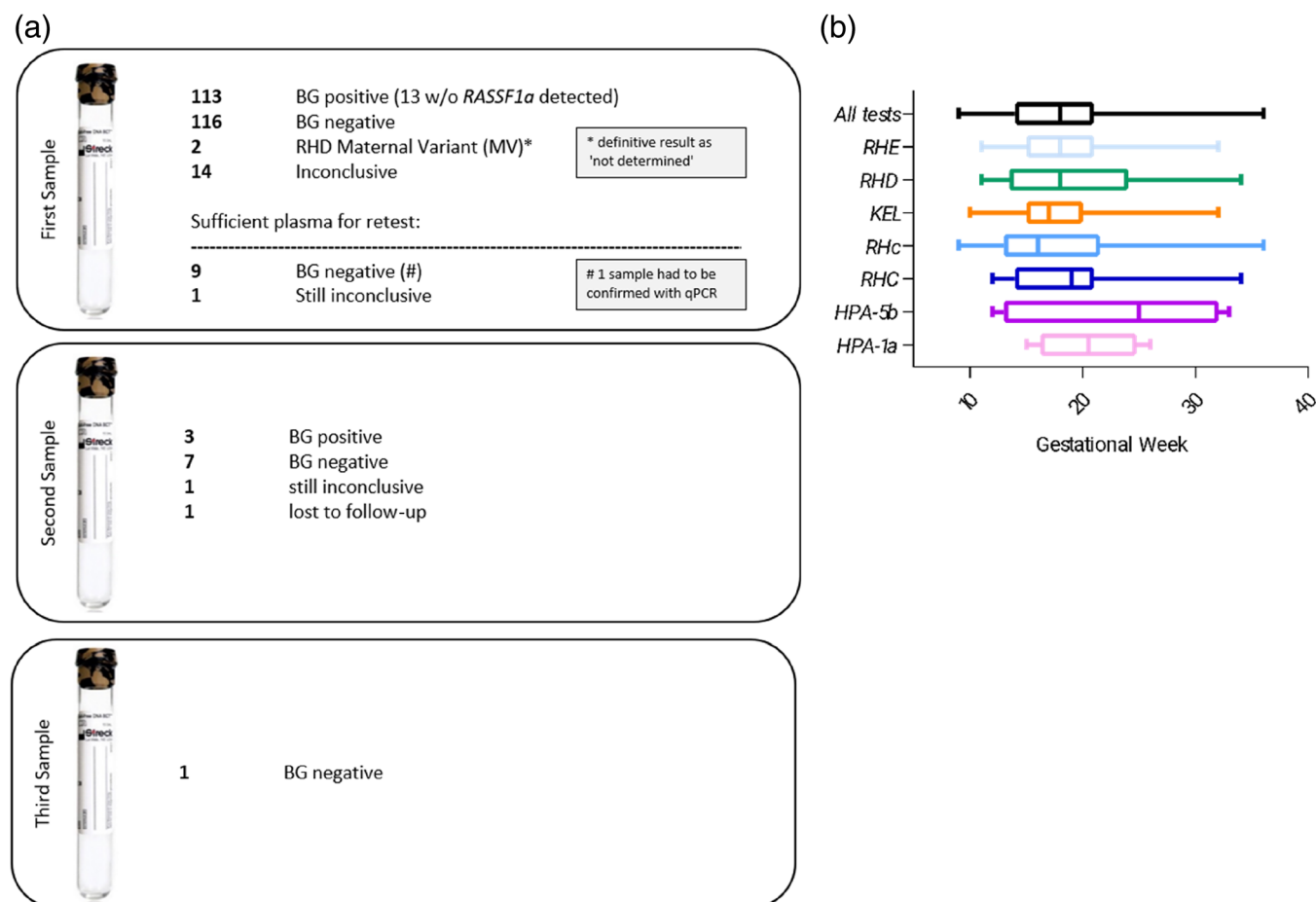


FIGURE 1 Clinical results foetal blood group typing via droplet digital polymerase chain reaction (ddPCR). (a) Flowchart summarizing results of foetal blood group genotyping via ddPCR. Top to bottom shows first, second and third sample collections. (b) Box plot depicting gestational age at time of blood collection, represented per antigen. Average gestational age at time of blood collection was 18 weeks (IQR 14–21). BG, blood group; RHE $n = 91$, RHD $n = 59$, KELL $n = 41$, RHc $n = 31$, RHC $n = 16$, HPA-5b $n = 8$, HPA-1a $n = 4$. qPCR, quantitative polymerase chain reaction.

First-draw sample performance

Two-hundred and thirty-one of 250 test requests (in 210 unique pregnancies) had a definitive result in the first test (116 neg, 113 pos, 2 maternal RHD variants). For the 10 out of 19 samples without test results, there was sufficient plasma for repeat testing (Figure 1a, Cases 12, 48, 64, 98, 125, 138, 148, 192, 210, 224), which resulted in a conclusive result, except for Cases 98 and 148 that was only further tested by qPCR (Table 1). Thus, using first-drawn samples, 94.8% (237 of 250) of test requests had the foetal blood group and platelet antigen genotyped ($n = 124$ neg, $n = 113$ pos, Table 1).

QC gates

Eighteen requests resulted in 21 QC gate failures on the first test on the first-drawn sample (see Method M1 in Supporting Information). QC failures were as follows: three cases with discordant *mRASSF1a* (Cases 138, 148, 194); two cases without *mRASSF1a* (Cases 58, 211);

four cases with discordant result for blood group and platelet antigen (Cases 12, 48, 98, 101); five cases with insufficient test material (Cases 39, 58, 160, 174, 178); one case with too much cfDNA input (Case 210); four cases with false negative or false positive controls (Cases 64, 192 and 125, 224 respectively); one case (Case 210) with insufficient droplets and one case (Case 148) with no positive foetal markers at GA21 and therefore deferred to qPCR (Table S2).

Second-draw sample performance

Twelve second samples were requested (Cases 39, 58, 71, 98, 101, 157, 160, 161, 174, 178, 194 and 211). Two of these were requested for confirmation only and gave the same result for K as their first sample. One of 12 cases (Case 98) did not send in a second-draw sample and was lost to follow-up. One second-draw sample (Case 211, GA19) still resulted in 'inconclusive' and required a third sample that was sent in GA24, in which eventually a negative foetal blood group (RHD) was diagnosed. In summary, the second testing

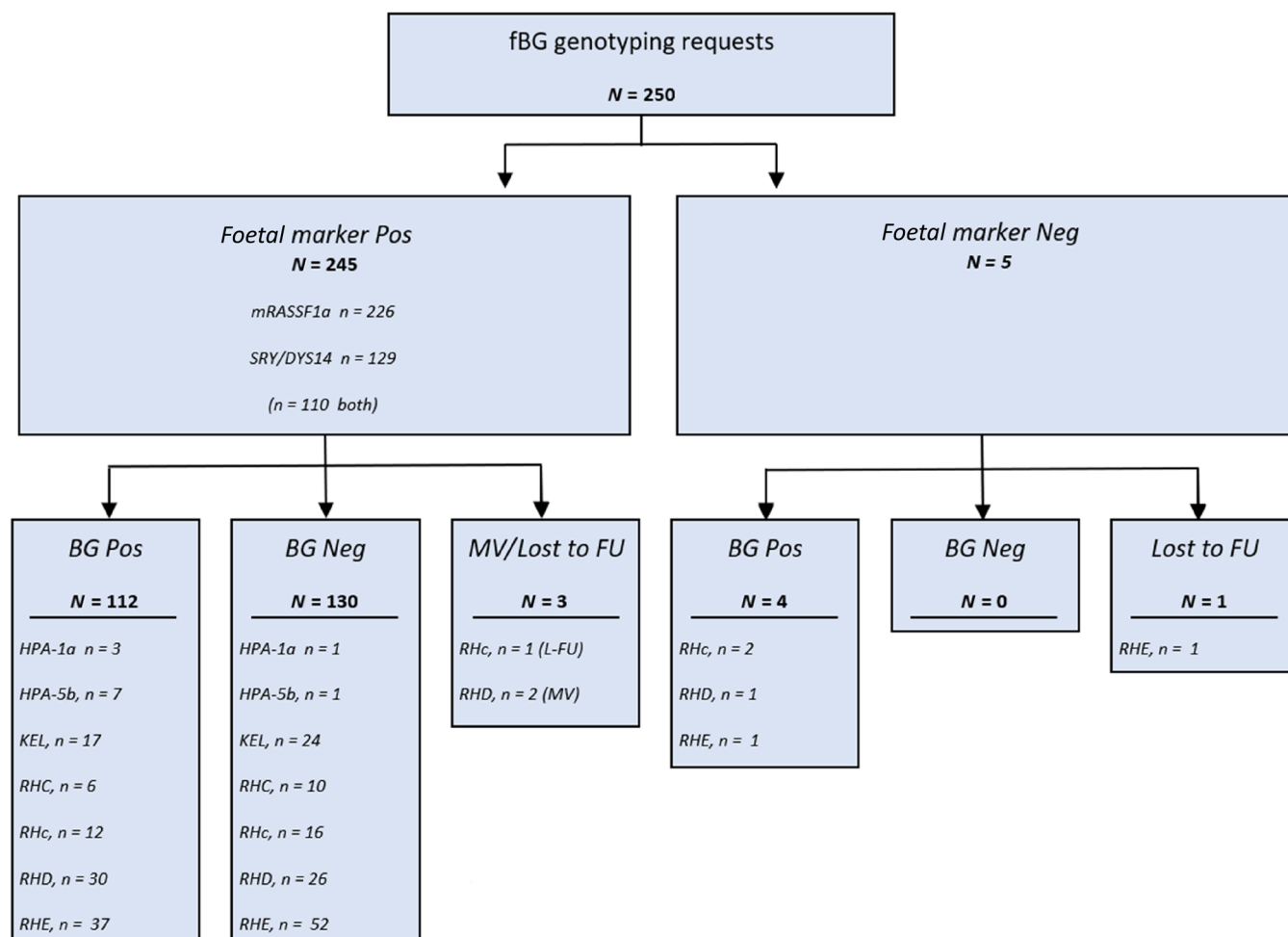


FIGURE 2 Test results for alloimmunized pregnant women tested with droplet digital polymerase chain reaction (ddPCR). BG, blood group; fBG, fetal blood group; Foetal blood group or platelet antigen, foetal blood group; FU, follow-up; MV, maternal D-variant; neg, negative; pos, positive.

round added eight final results (Case 39 *RHE*-pos, Case 58 *K*-neg, Case 71 *RHC*-pos, Case 101 *RHD*-neg, Case 160 *RHC*-neg, Case 174 *RHC*-pos, Case 178 *RHD*-neg and Case 194 *RHE*-neg) (Table 1).

Foetal DNA controls

Inclusion of the foetal markers *mRASSF1a* and *SRY/DYS14* as QC gating is crucial for the reliability of the reported results. *mRASSF1a* was positive in 88.2.5% (202 of 229 samples) tests in first received samples and in 54.5% (6 of 11) of second samples and 100% (1 of 1) the third sample. A positive ddPCR *mRASSF1a* provided confidence for 122 of the 131 (93%) negative foetal blood group and platelet antigen results. Foetal genotyping in eight other samples (Cases 62, 90, 91, 118, 144, 161, 194 and 206) relied solely on positive *SRY/DYS14* (Figure 3), while for one sample (Case 148) qPCR was used. GA was significantly shorter for cases ($n = 22$) with negative *mRASSF1a* in all of their repeats (median 16; IQR 13–19) as compared with those with positive *mRASSF1a* ($n = 195$ because GA unknown in $n = 12$ cases)

(median 18; IQR 14–22 weeks) tested by unpaired, two-sided *t*-test $p = 0.029$ (Table 2). Among these 22 *mRASSF1a*-negative cases, *SRY/DYS14* was detected in 17.

Reporting without any foetal markers

Four cases had their blood group and platelet antigens genotyped positive in absence of *mRASSF1a* and *SRY/DYS14* in any of their repeats (Case 56 [*RHD*-pos], Case 174 [*RHC*-pos], Case 185 [*RHE*-pos] and Case 221 [*RHC*-pos]). Case 148 could not be called because of the absence of foetal markers despite being clearly *RHE*-negative in ddPCR and was later confirmed *RHE*-negative in qPCR.

Maternal RHD variants

In two cases, foetal blood group and platelet antigen genotype could not be not determined because of a maternal *RHD* variant. One

TABLE 1 Testing flow.

Allele	HPA-1a			HPA-5b			K1			RHc			RHD			RHE			ddPCR result N; (cumulative %)
	Neg	Pos	Incon	Neg	Pos	Incon	Neg	Pos	Incon	Neg	Pos	Incon	Neg	Pos	Incon	Neg	Pos	Incon	
First draw, first test	3	1		1	7		19	17	5	14	12	5	10	6		22	31	2	231; (92.4)
First draw, second test							4			1		1		1					8; (95.6)
Second draw, first test							1			1	2		1		1	1	1		8; (98.8)
Third draw, first test													1						1; (99.2)
Totals	3	1		1	7		24	17		16	14		10	6		26	31	2	248; (99.2)

Note: Summary of 250 foetal blood and platelet antigen typing results grouped by predicted antigen (columns) and results during subsequent stages of testing (rows). Totals in red never had a foetal blood and platelet antigen result. Definitive result column includes the two maternal RHD variants.

Abbreviations: ddPCR, droplet digital polymerase chain reaction; FU, follow-up; incon, inconclusive; MV, RHD maternal variant detected; neg, negative foetal blood and platelet antigen genotype of interest diagnosed; pos, positive foetal blood and platelet antigen genotype of interest diagnosed; qPCR, quantitative polymerase chain reaction.

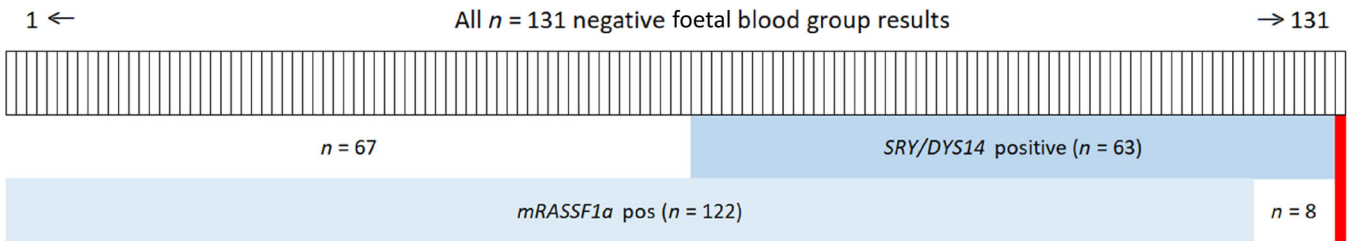


FIGURE 3 Positive foetal marker distribution in all $n = 131$ negative blood and platelet antigen genotyped cases. Note that one test (red, Case 148) was performed by quantitative polymerase chain reaction (qPCR) after failing once in droplet digital polymerase chain reaction (ddPCR). *mRASSF1a*, methylated *RASSF1a*; *SRY*, sex-determining region of Y.

TABLE 2 Distribution of *mRASSF1a* positivity versus gestational age.

	<i>n</i> (pregnancies)	Min GW	Max GW	Mean GW	Median GW	H_0 : GA are the same
Cases never <i>mRASSF1a</i> positive	22	11	21	16	16	Unpaired, two-sided t-test; $p = 0.029$
Cases ever <i>mRASSF1a</i> positive	207	9	36	19	18	
	229					

Note: Twenty two cases had no positive *mRASSF1a* detection and correspond to significantly shorter gestational age when compared with all other 207 cases.
Abbreviations: GA, gestational age; GW, gestational week; H_0 , null hypothesis; max, maximum; min, minimum; *mRASSF1a*, methylated *RASSF1a*.

mother (Case 18) had *RHD**03.03/01N.01 (DIIIC) detected with multiplex ligation-dependent probe amplification (MLPA), and the other (Case 21) had *RHD**01N.72/01N.01 (compound heterozygote null) detected by MLPA while the father carried three *RHD* variants on two different alleles (detected with Sanger sequencing) (Table S2).

DISCUSSION

We report the ddPCR results of non-invasive foetal blood group and platelet antigen genotyping for *RHD* (RH1), *RHE* (RH3), *RHC* (RH4), *RHC* (RH2), *K* (K1), *HPA-1a* and *HPA-5b* in alloimmunized pregnant women. In 1 year, a total 250 foetal blood group and platelet antigen genotype requests in 229 alloimmunized pregnant women were successfully tested in 98.4% (246 of 250). Unsuccessful cases included two in which maternal *RHD* variants precluded foetal genotyping and two lost to follow-up. Eight (3.3%) (8 of 246) results were obtained only after request of a second sample. Note that Cases 157 (GA14, K-pos) and 161 (GA15, K-neg) had successful ddPCR results in their first-drawn sample and were confirmed for information purposes only, at GA20 and GA18 with the same results. We included *SRY/DYS14* and *mRASSF1a* to the ddPCR as important foetal controls for reporting negative foetal genotypes. With 98.4% success rate, our ddPCR assay is an improvement over previous 97% success rate of qPCR method and no longer depends on multiplex in/del PCRs, which in about 10% of the cases remained inconclusive and were highly labour-intensive [7]. The inclusion of foetal marker *mRASSF1a* to *SRY/DYS14* increased definitive diagnostic calls in a further 6% of cases. Tests with inconclusive *mRASSF1a* marker were observed in 7.6% (19 of 250 tests). As expected [10], we observe that cases with negative *mRASSF1a* in

the first tested samples are associated with significantly lower GA compared with samples with positive *mRASSF1a*. Furthermore, we found that 12 of the 19 cases without *mRASSF1a* could be called because of a positive blood group or platelet antigen. Of the remaining seven with inconclusive *mRASSF1a*, six cases received a conclusive negative blood group or platelet antigen because *SRY/DYS14* was positive. In one case (Case 194), the blood group and platelet antigen ddPCR was discordant between extractions and was later diagnosed negative on a follow-up sample. To further improve the performance of our in-house-developed ddPCR test and reduce re-testing of samples, we will further automate the protocol with the use of pipetting robots, starting from the cfDNA extraction process up to the data analysis. One explanation for the occasional combination of a positive blood group and platelet antigen concurrent with a negative *mRASSF1a* might be incomplete foetal cfDNA methylation. Although increased multiplexing with DNA methylation-independent polymorphic genomic targets might be possible, our ddPCR already has excellent performance. Requesting a repeat sample later in pregnancy is a more pragmatic solution, and was needed in only eight cases. In conclusion, our in-house-developed and validated ddPCR foetal blood group and platelet antigen typing assay demonstrated excellent real-world performance and is an improvement over RT-qPCR for determination of foetal blood group and platelet antigen genotypes in alloimmunized pregnant women. The assay provided a conclusive foetal blood group and platelet antigen result in 99% (247 of 250) of foetal blood group and platelet antigen requests with minimal repeat testing. In general, clinicians stop clinical monitoring if we report the foetus is negative for the implicated antigen. A midwife will continue to provide care to women with antigen-negative fetuses and will not refer such women to second-line (clinical) obstetric care.

We assume the reports were helpful for the obstetric care providers, also to make sure that appropriate care is provided to pregnancies with fetuses that are positive for the implicated antigen.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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