Bridging the gaps: prevention, management, and future perspectives in hemolytic disease of the fetus and newborn

Oever, R.M. van 't

II

HOW TO IMPROVE A SCREENING PLATFORM

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Routine use of a spike-in DNA control as process control for foetal *RHD* typing: real life performance of this canary

Renske M. van 't Oever
Heleen Woortmeijer
Ahmad Javadi
Aicha Ait Soussan
Barbera Veldhuisen
Claudia C. Folman
C. Ellen van der Schoot
Erik H. van Beers
Masja de Haas

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Abstract

Background and Objectives: Non-invasive foetal *RHD* (*fRHD*) genotyping is widely implemented to prevent unnecessary administration of antenatal anti-D prophylaxis. Reliable assay performance is critical. In line with expert recommendations, we validated and implemented an artificial spike-in extraction control in our previously published assay. In this study, we report on assay verification and its performance in a 2-year cohort.

Study Design and Methods: *fRHD* typing was performed with cell-free DNA isolated from maternal plasma from gestational age week 27 or later. A circular plasmid with a fragment of glycoprotein B gene of the Phocid herpes virus type 1 (PhHV1-gB) (spike-in control) was added to the plasma before DNA extraction. Assay accuracy was verified with gestational week-27 plasma samples and corresponding cord blood samples from D-negative pregnant women. In addition, assay performance over time was evaluated in a 2-year cohort.

Results: The performance verification of our modified assay showed no false negative and one false positive test result in a small clinical cohort (n = 191). In a further 47,391 samples across 1111 runs, we observed eight false negative results due to technical failures that were prevented by the addition of the spike-in control. In this larger series, the spike-in control was the sole detector of a technical problem most likely related to different batches of the DNA extraction kit.

Conclusion: This study demonstrates the prevention of false negative *fRHD* typing results by the addition of an artificial extraction control. This control allows improved monitoring of assay performance, thereby ensuring assay consistency. Findings underscore the importance of thorough quality assurance measures in *fRHD* genotyping.

Highlights

- We evaluated the reliability of a foetal *RHD* (*fRHD*) genotyping assay and showed that this was enhanced through a spike-in DNA extraction control.
- This enhancement in diagnostic accuracy allowed the prevention of four false negative test results.
- The spike-in control also provided timely detection of reagent-linked performance issues

Introduction

Haemolytic disease of the foetus and newborn (HDFN) is mostly caused by maternal antibodies against the D antigen of the RH blood group system and can be life-threatening.[1] Introduction of postnatal anti-D immunoglobulin prophylaxis (RhIg) in the 1960s and antenatal prophylaxis in the 1990s reduced alloimmunization rates from 15%–20% to ~0.5%.[2-4] To target antenatal RhIg only for women pregnant with RhD positive foetuses, many centres predict foetal D-positivity by *RHD* genotyping using maternal plasma–derived cell-free foetal DNA (cff-DNA).[3-5] In 2016, we reported the performance of our in-house developed, fully automated assay in 32,222 D-negative pregnant women[6] using a 'no template control' as negative control and pooled plasma in two different dilutions from pregnant D-negative women as positive control.[6] We concluded that our assay at 27 weeks of gestation was highly reliable and could serve as a single test to guide both antenatal and postnatal RhIg use, with acceptable false positive and false negative results.[6] However, we observed three out of nine false negative results due to technical failures. Since we obtained cord blood samples for 80% of cases, this would imply an expected three false negative cases due to technical failures on an annual basis.[6] Previously, we had proposed that for screening purposes, the absence of a control for the presence of foetal DNA was acceptable and that the addition of in-process controls would depend on the associated extra costs.[7] In 2022, an expert group from the cf-DNA subgroup of the International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology (RCIBGT) published recommendations on validation and quality assurance for non-invasive prenatal testing of foetal blood groups, including the recommendation to add an extraction and amplification control.[8] Here we report the performance of our modified foetal *RHD* (*fRHD*) assay with, as in-process control, a cloned fragment of the glycoprotein B gene of the Phocid herpes virus type 1 (PhHV1-gB) (spike-in control) added to the plasma sample. We present the results obtained with a verification cohort of 191 paired maternal week-27 plasma samples and cord blood samples and reviewed test performance with focus on the prevention of false negative results in the first 2 years after implementation of the optimized assay.

Materials and methods

Setting

fRHD testing between weeks 27 and 29 of pregnancy and provision of RhIg prophylaxis in week 30 and after birth are part of the antenatal screening programme offered to all D-negative pregnant women in the Netherlands (~24,300/year)[9] by the National Institute for Public Health and the Environment (RIVM) on behalf of the Minister of Health, Welfare and Sport.[10] *fRHD* testing is performed by Sanquin Diagnostic Services in Amsterdam. For testing, 9 mL of EDTA blood is drawn. This blood sample is transported

at room temperature either by surface mail or by Sanquin's courier service and should be processed within 5 days. Haemolytic samples (visual inspection) are not processed.

Data collection and ethical permission

For clinical verification, a multicentre study was conducted, collecting an additional EDTA-blood sample at gestational week 27 and a cord blood sample after birth. This collection provided a cohort of 191 cases.

If false positive or false negative *fRHD* PCR cases were identified based on cord blood serology, *fRHD*-, mRASSF1a- and DYS14/SRY-specific ddPCRs[11] were performed on DNA isolated from stored plasma samples, using protocols applied for foetal genotyping in alloimmunised pregnancies. Nineteen midwifery practices participated in recruiting pregnant women. The study was approved by the Leiden University Medical Center Medical Ethics Committee (METC-LDD, study registered as P21.035), and all participants provided written informed consent.

Furthermore, we reviewed the anonymised data obtained for all *fRHD* tests registered as part of the national screening program in the period 15 February 2022 to 16 February 2024, directly after implementation of the spike-in control (n = 47,391). This analysis constituted an evaluation of our internal process, and therefore did not require separate ethical clearance.

Pre-analytics in routine operation

Plasma isolation was robotically performed as described previously.[6] To all wells of a 96-wells plate using a Xiril (Xiril, Hombrechtikon, Switzerland) or Hamilton Microlab Star (Hamilton, Hamilton Bonaduz AG, Switzerland) robot, a 20-µL aliquot of a solution of 3335 copies/mL of the PhHV1-gB DNA plasmid and 1 mL of plasma were added. The spike-in PhHV1 control reagent (referred to as spike-in control)—a plasmid DNA of an 89-bp cloned fragment of the gB gene of the PhHV1-gB in a pMA-RQ vector (Life technologies)—has been validated analytically for stability (data in Supporting information and Figures S1–S5). An independent validation cohort of 274 clinical samples established the spike-in threshold used in daily practice after implementation.

This plate was then processed with the MagNa Pure 96 Instrument (Roche Holding, Basel, Switzerland) for DNA extraction, using the Viral NA Large Volume Kit (Roche), with a final elution volume of 50 µL.

Real time PCR analysis

Triplex real-time quantitative PCR (qPCR) analysis for non-*RHD*-pseudogene amplifying sequences of *RHD* exons 5 and 7 was performed as published before, with minor modifications (see Supporting information and Table S1) and the insertion of the

spike-in control [6]. The total reaction volume was 25 µL, containing 10 µL of pre-mixed TaqMan Fast Advanced Master Mix (Life Technologies) containing all primers and probes (Table S1) and 15 µL of plasma-derived DNA. Primers and probes are used at final concentrations of 300 and 100 nM, respectively.

PCR conditions were as described previously[6], and the StepOne-Plus Real-Time PCR system with software v2.3 (Applied Biosystems, Foster City, CA, USA) was used for amplification. Pre-analytical analysis concerning the addition of the spike-in control (methods described in Supporting Information and Figures S1–S5) showed that this had no effect on the Ct values of the exon 5 and exon 7 PCR (Figure S6) nor on the limit of detection (4.2 and 4.6 pg, corresponding to about 1 geq, Supporting information and Figure S3).

Quality Control

An individual foetal typing result was considered valid if at least two out of three spike-in control replicates had Ct values below 35.73, the upper limit, which was based on the mean + 2 × SD (1.0) in the validation series (n = 274). If the spike-in control failed, the PCR amplification plots were reviewed and the PCR assay volume was checked for inconsistencies to conclude on the cause of assay failure. Test runs were performed with 48-well cff-DNA extraction plates accommodating two controls and up to 46 samples. Each run contained a positive control (run control, RC) made from pooled 27-week plasma samples from at least 80 D-negative pregnant women and the first run of every day contained a daily control (DC) which was a twofold dilution of the RC. Run results were considered valid if the non-template control (NTC) was negative and the positive control was within specifications.

The RC was accepted when two out of three replicates were positive with Ct values of 32.03–35.08 for exon 5 and 32.44–36.05 for exon 7. Specifications for the DC were as follows: two out of three replicates were positive with Ct values of 32.52–36.90 for exon 5 and 32.72–38.06 for exon 7. Repeating rules were as follows: if either the RC or the DC was out of specifications, all samples with a negative *fRHD* typing result of the impacted run were repeated. If the spike-in control was above 35.73 in at least two out of three replicates, *fRHD* negative samples were repeated. Inconsistent results (weak positive Ct values and one of three positive for either exon of *RHD*) were also repeated.

An automated interpretation algorithm was used based on the number of foetal signals for both *RHD* exons (six replicates) per sample (Table S2). An *RHD* exon 5 and *RHD* exon 7 PCR with Ct value below 30 represents maternal *RHD*-derived signals obscuring a potential foetal signal, precluding a conclusion on the foetal genotype status and therefore classifies as undetermined. Ct values between 30 and 40 represent *fRHD*-derived DNA

and Ct values above 40 represent negative. If the three signals for an exon split between three categories (maternal, foetal and negative), the result is inconclusive (Table S2).

Statistical analysis

For the clinical verification, *fRHD* genotyping results were compared with the reference standard (cord blood), and sensitivity, specificity, false negative rate, false positive rate, positive predictive value, negative predictive value and proportion of technical failures were calculated.

For the 2-year cohort analysis, we determined how often results were invalid due to RC or DC failures, or failure of the spike-in control. These repeat frequencies were benchmarked against a 0.46% repeat rate obtained from a large independent dataset (historical data [n = 48,786] calculated over a 2-year period [2017–2018] prior to implementing the spike-in control; data not shown).

Furthermore, we cross-compared the outcome to preanalytical conditions such as sample age at the time of plasma separation and gestational age at venipuncture.

Comparisons were made using an analysis of covariance (ANCOVA) implementation in Python (using Pingouin as part of SciPy 1.13.1). Continuous dependent variables were tested with continuous as well as categorical variables in the context of one or more covariates. Sum of squares (SS), degrees of freedom (DF), F-values (F), uncorrected p-values (unc p) and effect sizes (np2) were recorded. The significance threshold was set to 0.05.

An ANCOVA was performed to quantify the variation of individual reagent components in relation to the observed variability in the Ct values of the spike-in control. All ANCOVAs were controlled for the other two reagent lots (categorical) as well as for *RHD* exon 5 Ct value (continuous), *RHD* exon 7 Ct value (continuous), sample age (time elapsed until extraction; continuous), gestational weeks (continuous) and the day of the week of plasma centrifugation (Mon, Tue, Wed, Thu, Fri, Sat; categorical).

Data analysis was performed in Python v3.9 with Jupyter Lab v4.0, Statsmodels v0.14.2, SciPy v1.13.1, Numpy v1.26.4, Pandas v2.2.2 and Matplotlib v3.9. All computations were performed in Jupyter Notebook on a Win10 64-bit CPU using Anaconda v2.1.0.

Results

Clinical verification of the modified *fRHD* assay

For 195 D-negative pregnant women, the D typing of the cord blood samples was compared with the results of the week-27 *fRHD* (Table 1). Four cord blood samples were excluded

because of haemolysis (n = 3) or incorrect labelling (n = 1). There were no false negative results and there was one false positive result. Repeated *fRHD* typing of this sample showed a negative result, and it was therefore concluded that contamination in the initial assay was the most likely explanation. The false positive rate was 1.6% and the specificity 98.39%. The positive predictive value was 99.23% and the negative predictive value was 100%.

Table 1. Comparison of antenatal foetal genotyping results with cord blood typing in the same pregnancy.

	fRHD positive (n)	***fRHD negative (n)***	**Total (n)**
Cord blood D positive	129	0	129
Cord blood D negative	1	61	62
Total	130	61	191

Note: Foetal RHD PCR results of the week 27 sample is set out against the cord blood sample results from the same pregnancy.
Abbreviations: fRHD, foetal *RHD* genotyping*; n,* number.

Reported *fRHD* results in a 2-year real life performance cohort

In this study, 1111 runs were performed, totalling 47,391 unique samples. Of the 47,391 cases, 17,772 (36.5%) are reported as *fRHD* negative, 29,451 (62.1%) as *fRHD* positive and 168 (0.35%) as undetermined.

Repeat testing

The frequency of repeat testing in this 2-year performance cohort was 1.76% (n = 833/47,391; Table 2). In a 2-year cohort taken before the introduction of the spike-in control, repeat testing was 0.46% (n = 222/48,786; cohort from 1 January 2017 and 31 December 2018). Repeat testing was counted successful when obtaining a conclusive result.

We distinguished 12 different reasons for retesting (listed as Cat 1–12 in Table 2). The first six related only to the spike-in control, as in these runs the RCs, positive and negative controls were all within specification. Across all categories, retesting caused 13.2% (110/833) updated test results (Table 2). Of the total of 833 repeats, 430 (Cat 1–6; 51.6%) would presumably not have been repeated in absence of the spike-in, potentially missing 31 changes in test results; eight of these changing a negative result to a positive result (prevent potential false negative reporting), one positive to negative result and 22 undetermined to determined results (Table 2). In an additional 314 cases, the spike-in control served as an additional warning besides other quality control (QC) gates being out of specifications (Cat 9 and 10). In these cases, 14 test results were changed: 9 from negative to positive, and 5 from positive to undetermined.

Table 2 Comparison between initial and issued *fRHD* results of all (*n*=833) retested cases.

Category	Repeat Cause	Repeat Action Taken	Pos/ Pos	Neg/ Neg	Neg/ Pos	Pos/ Neg	Pos/ Und	Und/ Neg	Und/ Pos	cases (n)	% of 833
1	Spike-in Ct value (mean) aberrant compared to rest of plate (too high)	Repeat affected sample(s)	0	1	1	0	0	0	0	2	0.2%
2	Spike-in Ct value (mean) too high/ negative: no eluate added	Repeat affected sample(s)	0	3	2	0	0	0	0	5	0.6%
3	Spike-in Ct value (mean) too high/ negative: no mastermix added	Repeat affected sample(s)	4	7	2	1	0	11	11	36	4.3%
4	Spike-in Ct value (mean too high/ negative: no plasma pipetted	Repeat affected sample(s)	0	0	1	0	0	0	0	1	0.1%
5	Spike-in Ct value (mean) too high variation in many samples in the plate	Repeat selected samples: high spike-in & *fRHD*-neg + weak positives	31	348	2	0	0	0	0	381	45.7%
6	Spike-in Ct value (mean) aberrant compared to rest of plate (too low)	Repeat affected sample(s)	3	2	0	0	0	0	0	5	0.6%
7	Low volume warning (MP96)	Repeat affected sample(s)	0	3	0	0	0	0	0	3	0.4%
8	Leaked plasma warning (MP96)	Repeat affected sample(s)	1	0	1	1	0	0	0	3	0.4%
9	Spike-in Ct values too high + RC out of specs	Repeat entire run	159	83	6	0	3	0	0	251	30.1%
10	Spike-in Ct values too high + RC out of specs	Repeat *fRHD* negatives + weak positives	5	53	3	0	2	0	0	63	7.6%
11	Poor triplicate repeatability: *fRHD* 2/3 > Ct35 & < Ct40 for 1 or 2 exons	Repeat affected sample(s)	12	3	0	60	0	0	0	75	9.0%
12	*RHD* PCR amplification aberrant	Repeat affected sample(s)	0	5	1	2	0	0	0	8	1.0%
		totals	215	508	19	64	5	11	11	833	
		% of 833	25.8%	61.0%	2.3%	7.7%	0.6%	1.3%	1.3%		100.0%

Note: Reasons for repeating the fRHD test (rows), repeat actions and retest consequence on fRHD results (columns).
Abbreviations: fRHD, foetal *RHD;* MP96, MagNa Pure 96; RC, run control

Technical errors

Figure 1 shows the detection of a sudden high variability in the spike-in Ct values from August 2022 until April 2023 (for the complete dataset of the study period, see Figure 2). As depicted, the spike-in results were more affected than the *RHD* results. A subset analysis with tests performed in this period (n = 15,949 cases) showed an elevated repeat frequency of 4.51% (720/15,949) compared with 0.30% samples before this time period (37/12,242) and 0.40% after this time period (76/19,200) (Table S3). Excluding this 8-month period, repeat testing during the 2-year study period was 0.35% (n = 113/31,442). The manufacturer of the DNA extraction kit informed us after ample root cause analysis that the assay variation could have been caused by unintended higher levels of cations present in the eluate in specific lots of extraction kits.

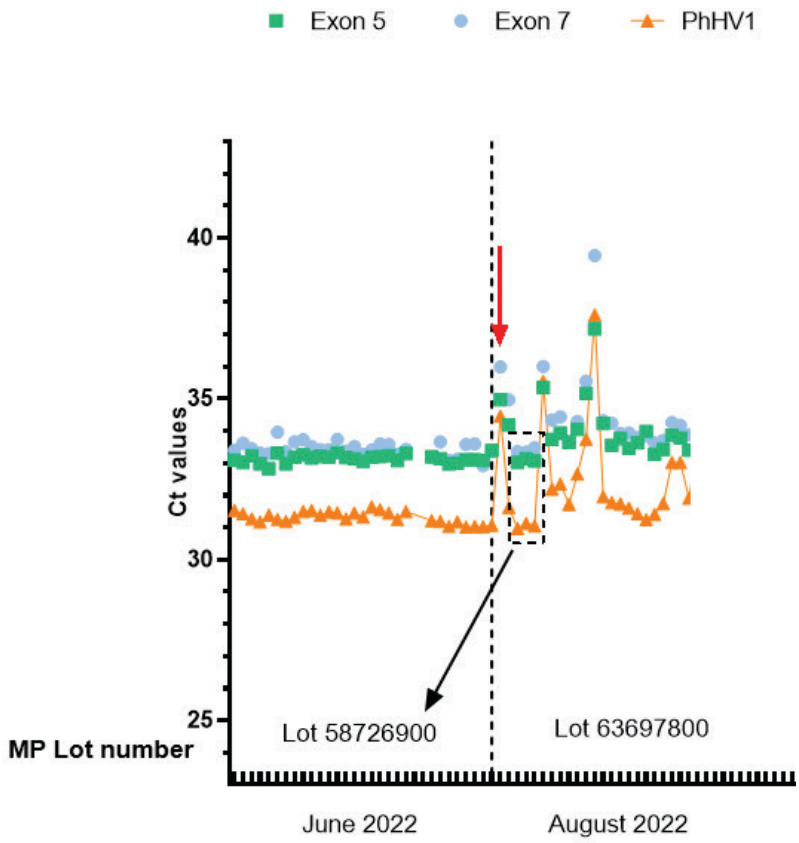

Figure 1 Mean Ct values of RHD Exon 5 (green squares) and 7 (blue circles) and spike-in control; orange triangles) per run over time in June and August of 2022. Lot numbers are separated by the black dashed line. The black outlined rectangle marks runs performed with the same DNA extraction kit as used in June 2022 as indicated. Red arrow indicates the first date with an observed effect of a failed run due to interfering substance. PhHV1, Phocid herpes virus type 1.

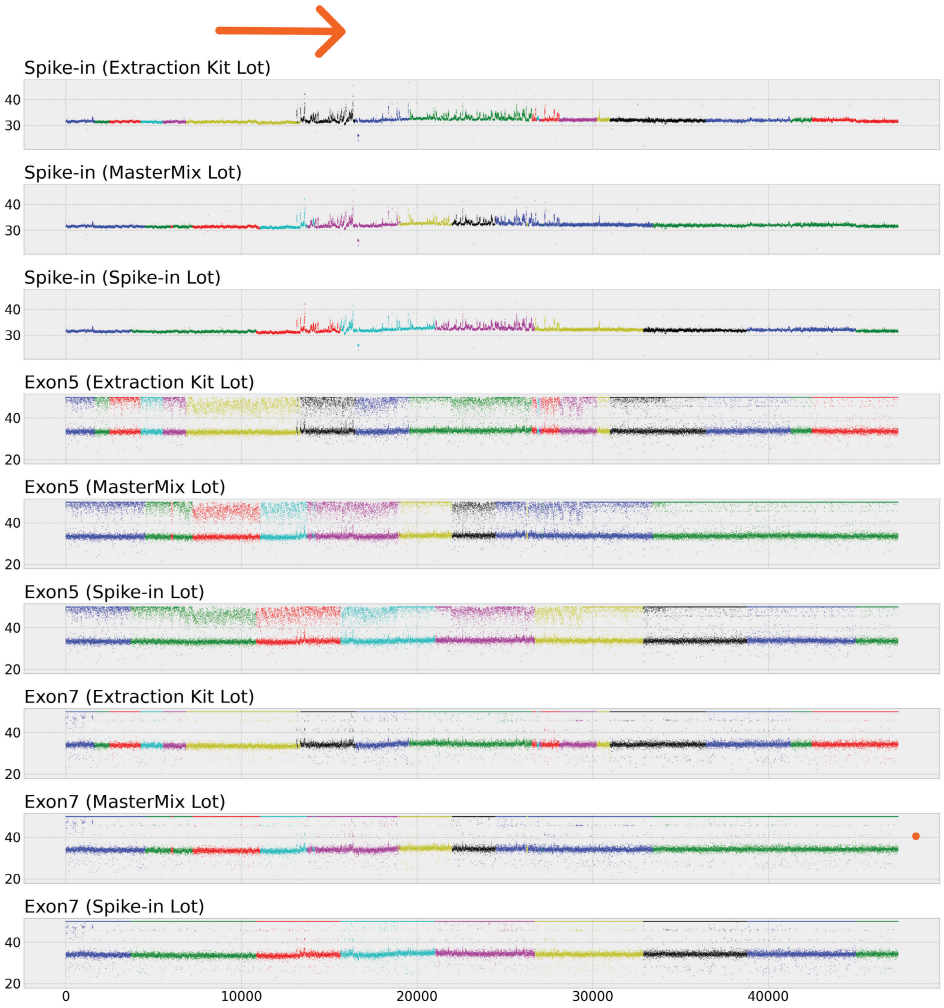

Figure 2 Longitudinal mean Ct values (mean of triplicate) n = 47,391; 15 February 2022 until 16 February 2024 (x-axis). The top three panels show spike-in mean Ct values (y-axis). The middle three panels show exon 5 mean Ct values, and the bottom three panels show exon 7 mean Ct values. There are three reagents for which subsequent lots are colour-indicated (blue, green, red, cyan, magenta, yellow, black). Beyond seven lots, colours are repeated for convenience. For Ct values of individual wells, see Figure S7. Orange arrow corresponds with period in Figure 1.

To investigate the factors influencing spike-in control Ct values, three ANCOVA analyses were performed on a dataset of 47,931 results (Tables S4–S6). The first ANCOVA examined the impact of the DNA extraction kitlot (kitlot) and found a significant association with spike-in control Ct values ($p < 10^{-6}$, $np2 = 0.238$). Among the seven other co-variables, five had minimal effect sizes (Table S4). The second ANCOVA analysed the effect of the

MasterMix lot and also showed a strong association ($p < 10^{-6}$, np2 = 0.26), with five of the seven co-variables having minimal impact (Table S5). The third ANCOVA assessed the spike-in lot (SIlot), revealing a significant effect ($p < 10^{-6}$, np2 = 0.13), while five co-variables again had minimal influence (Table S6).

In summary, the DNA extraction kitlot and MasterMix lot were the strongest contributors to variation in spike-in control Ct values. Additionally, the ANCOVA models as applied to the entire dataset of 47,391 samples confirmed that *RHD* Ct values were independent of spike-in control Ct values, as shown by the minimal effect sizes of exon 5 and exon 7 (Tables S4–S6).

Proportions of negative samples in assays with high spike-in control Ct values are unchanged

We retrospectively demonstrated that the proportion of negative results of 36.9% during a period with increased frequency of high spike-in Ct values was not elevated as compared to before (37.6%) and after (37.9%) (Table S7).

Recommended quality assurance results

Following the recommendations in Clausen et al.[8], the interval of time between venipuncture and separation of plasma from blood cells should be as short as possible when using EDTA tubes and should preferably not exceed 5 days. For 46,064 of 47,391 cases, the time between venipuncture and separation of plasma was recorded in days; 44,220 (96%) were separated within the recommended 5 days. Figure 3a shows no obvious correlation for *RHD* exon 5 (left), exon 7 (middle) or spike-in control (right) when Ct values are plotted against elapsed days, recapitulating previously reported shipping time robustness for samples collected in EDTA. Figure 3b shows no major correlation between *RHD* exon 5 (left), exon 7 (middle) or spike-in control (right) with the gestational age at the moment of drawing of the samples. The mean gestational age was 27.34 weeks with median 27. The far majority of samples were from a gestational age of 27 weeks as per protocol of the screening programme.

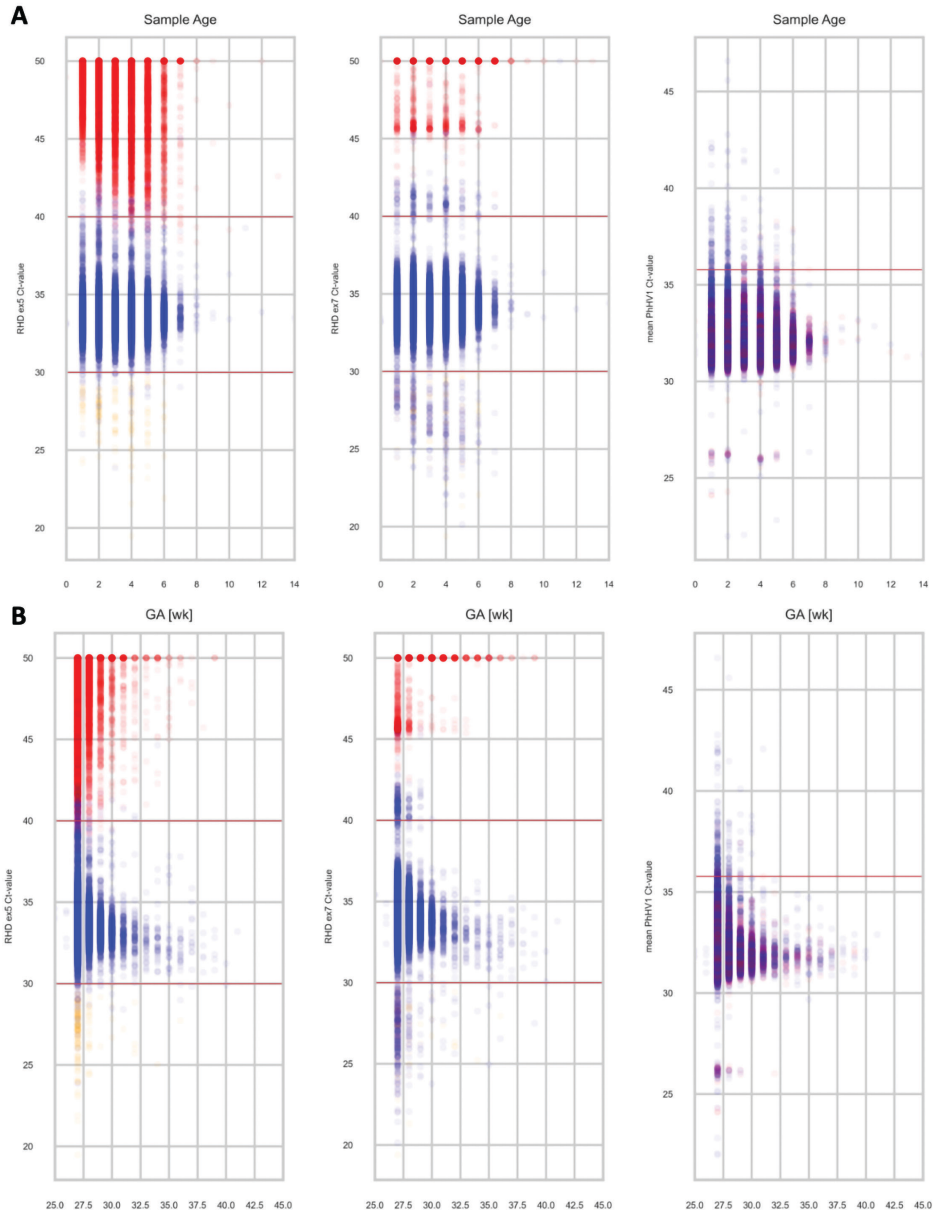

Figure 3. Stability of mean triplicate Ct values set out against gestational age and interval between blood draw and testing. (a) Sample stability. Ct values (mean of triplicate wells) for *RHD* exon 5 (left), RHD exon 7 (middle) and spike-in control (glycoprotein B gene of the Phocid herpes virus type 1 [PhHV1-gB]) (right) stratified versus sample age (test date − sample separation date [d]) of this cohort. (b) Gestational age. Ct values (mean of triplicate wells) for *RHD* exon 5 (left), *RHD* exon 7 (middle) and spike-in control (PhHV1-gB) (right) stratified versus gestational age [wk] of this cohort. In both figures, samples are classified by this assay as *RHD* negative (red), positive (blue) or undetermined (yellow). *fRHD*, foetal *RHD*.

Discussion

In the current study, we show that implementing a spike-in control—added to the plasma sample and aimed to confirm both successful DNA extraction and PCR amplification—is feasible and of additional value. In an already highly accurate fRHD typing platform, we observed that the risk of issuing false negative results could be further mitigated, especially given the sensitivity of this control for disturbances in PCR assay conditions. Therefore, the spike-in control served as a 'canary in the coal mine'. In total, we recognized in the 2-year review period 430 individual samples that had to be repeated because spike-in control was not within specifications (Cat 1–6); here we prevented eight false negative results. This reflects prevention of four false negatives in a year's cohort, as was also indicated by our previous study in which we compared *RHD* typing results with cord blood serology.[6] In an additional 314 samples (Cat 9–10), the spike-in control served—next to the standard controls—as an additional warning, adding to the prevention of nine other false negative results. There were no samples in which the standard controls indicated technical failures, and the spike-in controls were within specification.

More and more centres discontinue the practice of cord blood RhD serology to confirm the *fRHD* typing result obtained with cff-DNA isolated during pregnancy.[3, 12, 13] In a setting without verification with cord blood serology of the *fRHD* typing result, one needs to guarantee that one is not drifting in assay sensitivity and specificity. Continuous monitoring of the obtained test results is helpful. In the series here reported, the increased failure of meeting specifications of the spike-in control and the RC and DC to the upper limit of acceptance indicated that one of the reagents was causing technical errors. Close collaboration with the DNA isolation kit manufacturer led to early problem solving showing that an extraction and amplification control can further improve and monitor the assay performance. In some commercial assay designs, a human housekeeping gene (such as C-C chemokine receptor type 5 [CCR5] and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) are used; also, some other commercial test kits use artificial DNA added to the plasma sample.[12-17] A spike-in control has the advantage over a housekeeping gene because of a priori knowledge about its expected Ct value. In other set-ups using NGS sequencing, sequencing assays are added to be used for confirmation of a large enough foetal fraction to issue a test result, which seems also a valid approach. An example is the assay offered by BillionToOne, but they report 1.1% (171/15,500) samples failing QC for a result.[18]

Recommendations for non-invasive prenatal testing for foetal blood groups have been published [8]. cff-DNA has been well validated in pregnancies of at least 10 weeks. The interval between venipuncture and separation of plasma from blood cells should be as short as possible when using EDTA tubes and should preferably not exceed 5 days. In the current study, we found no difference in test performance when sample age increased well over the 5-day limit, which is standard operation procedure. When using a housekeeping gene as internal control, the risk of Ct values running out of specification as the samples ages is considerable because of maternal leukocyte DNA. In one such study, we found a resting rate of 1.12% based on Ct cut-off values not meeting the specifications.[12]

A limitation of the study is that we employed the initially selected high cut-off threshold for spike-in control performance. Consequently, it is possible that some cases with suboptimal DNA isolation or PCR amplification efficiency were not detected. Based on the current study, we should be able to calculate more stringent cut-off values for spike-in control results, which will further improve the continuous monitoring of assay performance.

In conclusion, the implementation of a spike-in control in our laboratory's *fRHD* typing workflow enhanced the robustness of process monitoring and served as the most reliable indicator of performance deviations during a period of unforeseen technical issues. This approach enabled rapid and effective troubleshooting, thereby safeguarding the overall test performance. The addition of a spike-in control in *fRHD* screening has already been advised by ISBT's expert group, and with this study we confirm the validity of this recommendation since we further could prevent the occurrence of false negative *fRHD* typing results. The observed repeat frequency in the test with the spike-in control was 0.35% compared with 0.46% before its introduction also suggests an improved confidence of test results.

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Supplementary materials

Validation of spike-in control

The effect of addition of the spike-in control on amplification of both *RHD* exons, and vice versa was determined. Impact on amplification of *RHD* exon 5 and exon 7 by addition of the spike-in PhHV 1-gB was tested with four different experiments.

1. To assess the impact of addition of the spike-in control (PhHV 1-gB) dilutions 0 to 100,000 copies/PCR were added to 100 ng genomic DNA of D-positive and D-negative individuals (Figure S1A-B). Observed efficiencies were 103%, 103% and 102% for D-positive + PhHV 1-gB, D-negative + PhHV 1-gB and, H2O + PhHV 1-gB respectively. For all linearity curves the correlation coefficient was > 0.99 (Fig. S1C).

2. To further assess the impact of addition of the spike-in control a fixed amount of PhHV 1-gB (100,000 copies/PCR) was added to variable concentrations (5 to 50,000 pg) of genomic DNA genomic DNA of D-positive and D-negative individuals, respectively. Amplification of the *RHD* exon 5 and 7 at decreasing DNA concentration showed linearity down to a concentration of 5 pg for both exons, and no amplification was observed with *RHD*-negative DNA in the presence of PhHV1-gB (Figure S2). Efficiency was 93% and 101% for exon 5 and 94% and 94% for exon 7, respectively with or without PhHV 1-gB. All samples, spiked with PhHV 1-gB, showed similar amplification for PhHV1-gB with mean (SD) values of 24.92 (0.36) and 25.05 (0.23) with both *RHD*-positive (Fig. S2A) and *RHD*-negative DNA, respectively. Spiking 100,000 copies of PhHV1-gB to variable *RHD*-positive genomic DNA input showed similar amplification of *RHD* exon 5 and 7 with and without PhHV 1-gB (Fig. S2B S2C).

3. The limit of detection of *RHD* was established by parallel runs with or without PhHV 1-gB in the presence of decreasing quantities of *RHD* positive DNA (13.2, 6.6 3.3 and 1.7pg/well). The LOD for *RHD* exon 5 and 7 with or without PhHV 1-gB is similar (4.2 and 4.6 pg, corresponding to about 1 geq). (Figure S3).

4. The recovery and repeatability of the assay was tested using PhHV 1-gB-spiked (3335copies/ml plasma) in plasma pool samples of D-positive cell free DNA in maternal plasma of D-negative pregnant women (n = 40) or D-negative cell free foetal DNA in maternal plasma of D-negative pregnant women (n = 24) . The recovery of the plasmid in these different plasma samples was highly reproducible, mean Ct (± SD) value for PhHV 1-gB was 32.16±0.28 (Figure S4).

5. Reproducibility was determined by PCR in D-positive plasma samples and D-negative plasma samples (n = 40) and D-negative plasma samples (n = 24) spiked with PhHV 1-gB at a fixed concentration of 3335 copies/ml (corresponding to 1000 copies/PCR well). The reproducibility of the plasmid in these different plasma samples was high, with mean Ct (± SD) for PhHV 1-gB of 32.16 ± 0.28. Also no effect was found of adding the plasmid on isolation of total cell free DNA as measured by the albumin PCR. (Figure S5)

Overview of supplementary Tables and Figures

Table S1: Primer and probe sequences used for triplex *RHD* and PhHV 1-gB and singleplex albumin qPCR.
Table S2: Lookup table to translate the nine Ct values produced from foetal *RHD* screening PCR to result interpretation.
Table S3: Proportions of 833 repeats before, during and after period with kit problems.
Table S4: ANCOVA table for independent variable DNA extraction Kit "kitlot"
Table S5: ANCOVA table for independent variable MasterMix "mmlot"
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Table S7: Proportion of issued *fRHD* results before during and after kit problems.
Figure S1: *RHD* amplification plots and spike-in standard curve
Figure S2: Amplification plots of spike-in and *RHD* standard curves
Figure S3: Determination of limit of detection (LOD) for triplex qPCR assay
Figure S4: Determination for recovery efficiency of the assay
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Figure S6: Addition of the spike-in does not impact *RHD* Ct values
Figure S7: Longitudinal Ct values (single wells) during clinical testing

Table S1 Primer and probe sequences used for triplex RHD and PhHV 1-gB and singleplex albumin qPCR.

Target	Oligo	Nucleotide sequence (5' → 3') *	Final PCR concentration (nM)	Volume (µl)	Reference
RHD	Ex7F/primer	G**G**GTGTTGTAACCGAGTGCTG	300	0.075	1
	Ex7R/primer	CCG**GCT**C**C**GAC**G**GTAT**C**	300	0.075	1
	Ex7/probe	FAM-A**G**CTCC**A**TCAT**GG**GCT**A-** MGB	100	0.025	4
	Ex5F/primer	CGCCCTCTTCTTGTGGATG	300	0.075	2
	Ex5R/primer	GAACA**C**GGCATTCTTCCTTT**C**	300	0.075	2
	Ex5/probe	VIC-CTGGCCAAGT**T**TCAA-MGB	100	0.025	4
PhHV-1 (*gB* gene)	PhHV F/primer	GGGCGAATCACAGATTGAATC	300	0.075	3
	PhHV R/primer	GCGGTTCCAAACGTACCAA	300	0.075	3
	PhHV/probe	NED-TTTTTTATGTGTCCGCCACCA-MGB	100	0.025	4
Albumin	AlbF/primer	TGAAACATACGTTCCCAAAGAGTTT	300		5
	AlbR/primer	CTCTCCTTCTCAGAAAGTGTGCATAT	300		5
	Alb/probe	FAM-TGCTGAAACATTCACCTTCCATGCAGA-TAMRA	100		5

An overview of the primers and probes used in our PCR screening set up is displayed. We use a dual-labelled probe 5'-VIC/3'-MGB for RHD exon 5, 5'-FAM/3'-MGB for RHD exon 7 and 5'-NED/3'-MGB for the spike-in control PhHV1 was designed using (ABI) Primer Express software (Applied Biosystems). *RHD-specific residues are underlined and bolded. References: 1: Rijnders et al.; 2: Finning et al.; 3: Niesters HG; 4: this study; 5: Verhagen et al.
PhHV1: Plasmid with 89bp insert of a part of the Phocine Herpesvirus type I -gB gene, ex: exon; F: forward; R: reverse; alb: albumin.

Table S2 Lookup table describing when the PCR is called Positive or Negative.

	Number of positive wells *RHD* exon 5			Number of positive wells *RHD* exon 7			*fRHD* Result
	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	
1	0	0	3	0	0	3	Negative
2	0	0	3	0	1	2	Negative
3	0	0	3	0	2	1	Positive
4	0	0	3	1	1	1	Repeat
5	0	0	3	0	3	0	Positive
6	0	0	3	1	2	0	Positive
7	0	0	3	1	0	2	Negative
8	0	0	3	2	0	1	Positive
9	0	0	3	2	1	0	Positive
10	0	0	3	3	0	0	Positive
11	0	1	2	0	0	3	Negative
12	0	1	2	0	1	2	Negative
13	0	1	2	0	2	1	Repeat
14	0	1	2	1	1	1	Repeat
15	0	1	2	0	3	0	Positive
16	0	1	2	1	2	0	Positive
17	0	1	2	1	0	2	Negative
18	0	1	2	2	0	1	Positive
19	0	1	2	2	1	0	Positive
20	0	1	2	3	0	0	Positive

Table S2. *Continued*

	Number of positive wells *RHD* exon 5			Number of positive wells *RHD* exon 7			*fRHD* Result
	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	
21	0	2	1	0	0	3	Positive
22	0	2	1	0	1	2	Positive
23	0	2	1	0	2	1	Positive
24	0	2	1	1	1	1	Positive
25	0	2	1	0	3	0	Positive
26	0	2	1	1	2	0	Positive
27	0	2	1	1	0	2	Positive
28	0	2	1	2	0	1	Positive
29	0	2	1	2	1	0	Positive
30	0	2	1	3	0	0	Positive
31	1	1	1	0	0	3	Positive
32	1	1	1	0	1	2	Positive
33	1	1	1	0	2	1	Positive
34	1	1	1	1	1	1	Positive
35	1	1	1	0	3	0	Positive
36	1	1	1	1	2	0	Positive
37	1	1	1	1	0	2	Positive
38	1	1	1	2	0	1	Positive
39	1	1	1	2	1	0	Positive
40	1	1	1	3	0	0	Positive
41	0	3	0	0	0	3	Positive
42	0	3	0	0	1	2	Positive
43	0	3	0	0	2	1	Positive
44	0	3	0	1	1	1	Positive
45	0	3	0	0	3	0	Positive
46	0	3	0	1	2	0	Positive
47	0	3	0	1	0	2	Positive
48	0	3	0	2	0	1	Positive
49	0	3	0	2	1	0	Positive
50	0	3	0	3	0	0	Positive
51	1	2	0	0	0	3	Positive
52	1	2	0	0	1	2	Positive
53	1	2	0	0	2	1	Positive
54	1	2	0	1	1	1	Positive
55	1	2	0	0	3	0	Positive
56	1	2	0	1	2	0	Positive
57	1	2	0	1	0	2	Positive
58	1	2	0	2	0	1	Positive
59	1	2	0	2	1	0	Positive
60	1	2	0	3	0	0	Positive
61	2	1	0	0	0	3	Positive
62	2	1	0	0	1	2	Positive
63	2	1	0	0	2	1	Positive
64	2	1	0	1	1	1	Positive

Table S2. *Continued*

	Number of positive wells *RHD* exon 5			Number of positive wells *RHD* exon 7			*fRHD* Result
	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	Ct <30	30 ≤ Ct ≤ 40	Ct > 40	
65	2	1	0	0	3	0	Positive
66	2	1	0	1	2	0	Positive
67	2	1	0	1	0	2	Positive
68	2	1	0	2	0	1	Positive
69	2	1	0	2	1	0	Positive
70	2	1	0	3	0	0	Positive
71	3	0	0	0	0	3	Positive
72	3	0	0	0	1	2	Positive
73	3	0	0	0	2	1	Positive
74	3	0	0	1	1	1	Positive
75	3	0	0	0	3	0	Positive
76	3	0	0	1	2	0	Positive
77	3	0	0	1	0	2	Positive
78	3	0	0	2	0	1	Positive
79	3	0	0	2	1	0	Positive
80	3	0	0	3	0	0	Positive
81	1	0	2	0	0	3	Negative
82	1	0	2	0	1	2	Negative
83	1	0	2	0	2	1	Positive
84	1	0	2	1	1	1	Repeat
85	1	0	2	0	3	0	Positive
86	1	0	2	1	2	0	Positive
87	1	0	2	1	0	2	Negative
88	1	0	2	2	0	1	Positive
89	1	0	2	2	1	0	Positive
90	1	0	2	3	0	0	Positive
91	2	0	1	0	0	3	Positive
92	2	0	1	0	1	2	Positive
93	2	0	1	0	2	1	Positive
94	2	0	1	1	1	1	Positive
95	2	0	1	0	3	0	Positive
96	2	0	1	1	2	0	Positive
97	2	0	1	1	0	2	Positive
98	2	0	1	2	0	1	Positive
99	2	0	1	2	1	0	Positive
100	2	0	1	3	0	0	Positive

Look-up table for all possible scenarios in *fRHD* genotyping. Ct-values < 30 are considered maternal signals, Ct-values 30-40 are considered foetal signals and Ct-values > 40 are considered negative.
Ct: cycle threshold value, pos: positive, *fRHD*: foetal *RHD*.

Table S3 Proportions of 833 repeats before, during and after period with kit problems.

Testing period	Before	During	After
samples tested	12242	15949	19200
Cat-1	0	1	1
Cat-2	0	3	2
Cat-3	0	1	35
Cat-4	0	1	0
Cat-5	0	374	7
Cat-6	3	1	1
Cat-7	0	2	1
Cat-8	0	3	0
Cat-9	0	63	0
Cat-10	0	251	0
Cat-11	28	20	27
Cat-12	6	0	2
sum	37	720	76
% of this period	0.30%	4.51%	0.40%
% of 833	4.44%	86.43%	9.12%

Table S4 ANCOVA table for independent variable DNA extraction Kit "kitlot"

	Source	SS	DF	F	p-unc	np2
0	kitlot	6902.653029	16	897.800272	0.000000e+00	0.237892
1	mmlot	33.757378	1	70.250978	5.367756e-17	0.001524
2	silot	60.097898	1	125.067063	5.363147e-29	0.002710
3	zw_duur	0.293100	1	0.609957	4.348080e-01	0.000013
4	weekday	0.508260	1	1.057717	3.037420e-01	0.000023
5	elapseddays	11.172036	1	23.249627	1.427318e-06	0.000505
6	mean5	25.922945	1	53.947089	2.093632e-13	0.001171
7	mean7	28.794036	1	59.921989	1.007000e-14	0.001300
8	Residual	22113.297343	46019	NaN	NaN	NaN

Results from the ANCOVA statistics with DNA extraction kit lot number as independent variable. Kitlot: kit lot number of the DNA extraction kit, mmlot: MasterMix lot number, silot: lot number of the spike-in control used, ga: gestational age, weekday: day of the week (mon-fri), elapseddays: days elapsed between blood draw and sample testing, mean5: mean ct value of the triple PCR reaction of exon 5, mean 7: mean ct value of the triple PCR reaction of exon 7, SS: sum of squares, DF: degrees of freedom, F: F-ratio, p-unc: uncorrected p values, NP2: effect size.

Table S5 ANCOVA table for independent variable MasterMix "mmlot"

	Source	SS	DF	F	p-unc	np2
0	mmlot	8638.750266	8	2057.386301	0.000000e+00	0.263404
1	kitlot	499.039418	1	950.801291	1.139174e-206	0.020239
2	silot	0.580257	1	1.105542	2.930587e-01	0.000024
3	zw_duur	0.132960	1	0.253324	6.147467e-01	0.000006
4	weekday	4.146918	1	7.900970	4.942918e-03	0.000172
5	elapseddays	21.171590	1	40.337445	2.156633e-10	0.000876
6	mean5	25.631467	1	48.834683	2.822480e-12	0.001060
7	mean7	27.731657	1	52.836097	3.683209e-13	0.001147
8	Residual	24157.820913	46027	NaN	NaN	NaN

Results from the ANCOVA statistics with MasterMix lot number as independent variable. Kitlot: kit lot number of the DNA extraction kit, mmlot: MasterMix lot number, silot: lot number of the spike-in control used, ga: gestational age, weekday: day of the week (mon-fri), elapseddays: days elapsed between blood draw and sample testing, mean5: mean ct value of the triple PCR reaction of exon 5, mean 7: mean ct value of the triple PCR reaction of exon 7, SS: sum of squares, DF: degrees of freedom, F: F-ratio, p-unc: uncorrected p values, NP2: effect size.

Table S6 ANCOVA table for independent variable spike-in control batch "SIlot"

	Source	SS	DF	F	p-unc	np2
0	silot	3469.775697	8	850.390264	0.000000e+00	1.287735e-01
1	kitlot	104.954561	1	205.782377	1.440908e-46	4.451006e-03
2	mmlot	603.170034	1	1182.623823	6.469674e-256	2.505048e-02
3	zw_duur	0.889207	1	1.743451	1.867088e-01	3.787743e-05
4	weekday	0.005387	1	0.010562	9.181433e-01	2.294816e-07
5	elapseddays	7.378780	1	14.467430	1.427882e-04	3.142261e-04
6	mean5	65.903330	1	129.215385	6.670202e-30	2.799523e-03
7	mean7	67.354120	1	132.059922	1.597934e-30	2.860975e-03
8	Residual	23475.010930	46027	NaN	NaN	NaN

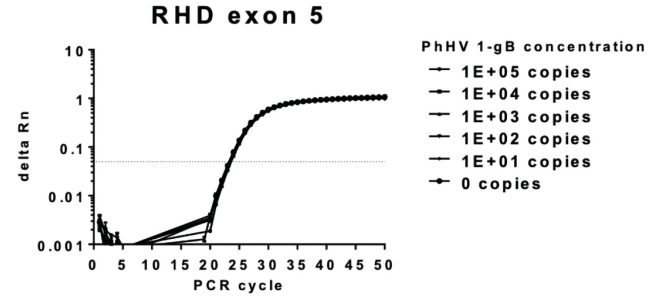
Results from the ANCOVA statistics with spike-in control batch lot number as independent variable. Kitlot: kit lot number of the DNA extraction kit, mmlot: MasterMix lot number, silot: lot number of the spike-in control used, ga: gestational age, weekday: day of the week (mon-fri), elapseddays: days elapsed between blood draw and sample testing, mean5: mean ct value of the triple PCR reaction of exon 5, mean 7: mean ct value of the triple PCR reaction of exon 7, SS: sum of squares, DF: degrees of freedom, F: F-ratio, p-unc: uncorrected p values, NP2: effect size.

Table S7 Proportions of issued fRHD genotypes.

	fRHD-neg	%	fRHD-pos	%	fRHD-und	%	total
Prior to high spike-in variation	4605	37.6%	7596	62.0%	41	0.3%	**12242**
Stratifed by spike-in control Ct < 35.73	4605		7596		41		
Stratifed by spike-in control Ct ≥ 35.73	0		0		0		
During high spike-in variation	5886	36.9%	10009	62.8%	54	0.3%	**15949**
Stratifed by spike-in control Ct < 35.73	5858		9770		53		
Stratifed by spike-in control Ct ≥ 35.73	28		239		1		
After high spike-in variation	7281	37.9%	11846	61.7%	73	0.4%	**19200**
Stratifed by spike-in control Ct < 35.73	7280		11838		73		
Stratifed by spike-in control Ct ≥ 35.73	1		8		0		
							47391

Displayed are the results of fRHD genotypes before, during and after the high spike-in control variation period. They are stratified by the spike-in cut-off Ct value of 35.73.

A



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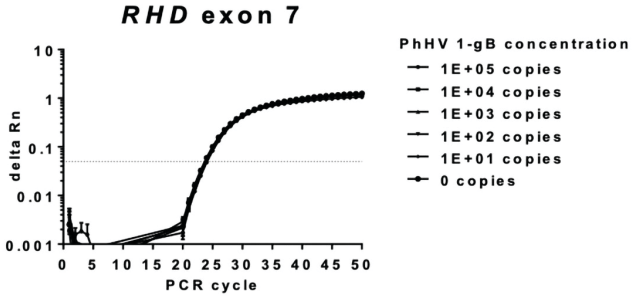


C

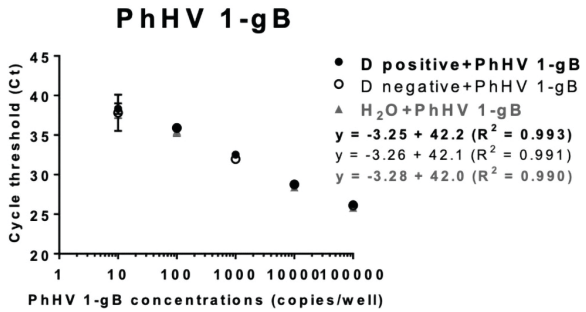
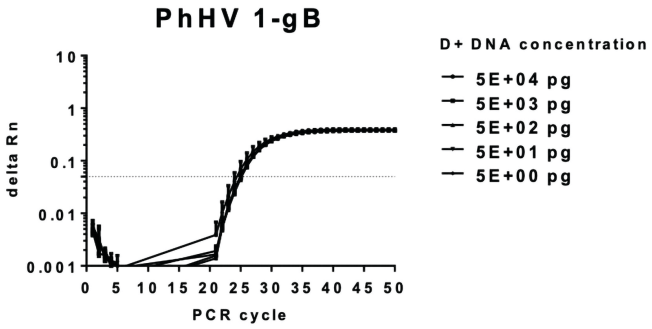
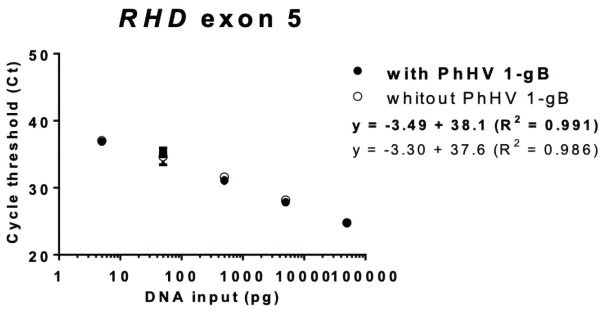


Figure S1 *RHD* amplification plots (A-B) and Spike-in standard curve (C) of triplex RHD exon 5 and 7 and PhHV 1-gB qPCR, using 10-fold dilutions of PhHV 1-gB in D-positive genomic DNA samples (100 ng/PCR well). The dotted line indicated the threshold line (0.05). PCR experiments were performed in duplicate.

●, D-pos + PhHV 1-gB, y = -3.25 + 42.2 (R2 = 0.993), efficiency 103%; ○, D-neg + PhHV 1-gB, y = -3.26 + 42.1 (R2 = 0.991), efficiency 103%; ▲, H_2O + PhHV 1-gB, y = -3.28 + 42.0 (R^2 = 0.990), efficiency 102%. The triplex qRCR assays were set up in a total reaction volume of 25 µL. Each final qPCR reaction well contained 12.5 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems), 100 ng of D-pos genomic DNA (4 µL/well) and 100 000, 10 000, 1000, 100 and 10 copies of PhHV 1-gB (5 µL/well). All primers and probes were used at final concentration of 300 and 100 nM.

A





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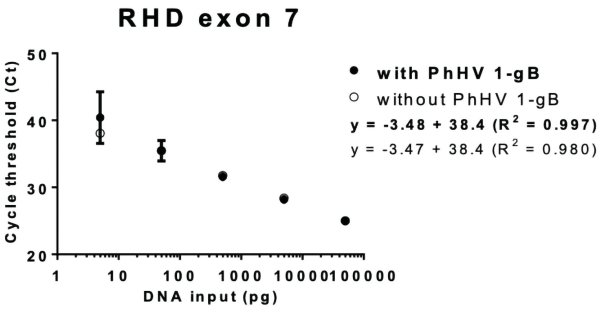

C

Figure S2 Amplification plots of Spike-in (A) and *RHD* standard curves (B-C) of triplex *RHD* exon 5 and 7 and PhHV 1-gB qPCR, using 10-fold dilutions of *RHD*-positive genomic DNA PhHV 1-gB (100,000 copies/PCR well). The dotted line indicated the threshold line (0.05). PCR experiments were performed in duplicate. ●, D-pos + PhHV 1-gB, y = -3.49 + 38.0 (R^2 = 0.991), efficiency 93% and y = -3.48 + 38.4 (R^2 = 0.997), efficiency 94%, for RHD exon 5 and exon 7, respectively. ○, D-pos - PhHV 1-gB, y = -3.30 + 37.6 (R^2 = 0.986), efficiency 101% and y = -3.47 + 38.4 (R^2 = 0.98), efficiency 94% for RHD exon 5 and exon 7, respectively**.** The triplex qRCR assays were set up in a total reaction volume of 25 µL. Each final qPCR reaction well contained 12.5 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems), 50 000, 5000, 500, 50 and 5 pg of genomic DNA (4 µL/well) and 100 000 copies of PhHV 1-gB (5 µL/well). All primers and probes were used at final concentration of 300 and 100 nM.

A

DNA input (pg)	% pos wells + PhHV 1-gB			% pos wells - PhHV 1-gB		
	***RHD* exon 5**	***RHD* exon 7**	**PhHV**	***RHD* exon 5**	*RHD* **exon 7**	**PhHV**
13.2	23/24(96%)	23/24(96%)	24/24 (100%)	23/24(96%)	23/24(96%)	0/24 (0%)
6.6	20/24(83%)	18/24(75%)	24/24 (100%)	20/24(83%)	17/24(71%)	0/24 (0%)
3.3	27/48(56%)	26/48(54%)	48/48 (100%)	26/48(54%)	22/48(46%)	0/48 (0%)
1.7	13/48(27%)	16/48(33%)	48/48 (100%)	14/48(29%)	18/48(38%)	0/48 (0%)

B

DNA input (pg)	Mean Ct (±SD) + PhHV1-gB			Mean Ct (±SD) - PhHV1-gB		
	***RHD* exon 5**	***RHD* exon 7**	**PhHV**	***RHD* exon 5**	***RHD* exon 7**	**PhHV**
13.2	35.01(0.54)	35.59(0.75)	31.27(0.27)	35.50(0.73)	35.78(0.94)	UD
6.6	36.01(0.69)	36.34(0.56)	31.20(0.18)	36.44(0.80)	36.74(0.69)	UD
3.3	36.04(0.66)	37.08(0.76)	31.20(0.27)	36.61(0.59)	37.31(0.60)	UD
1.7	37.55(2.40)	38.21(2.32)	31.15(0.27)	36.83(0.52)	37.86(0.55)	UD

C

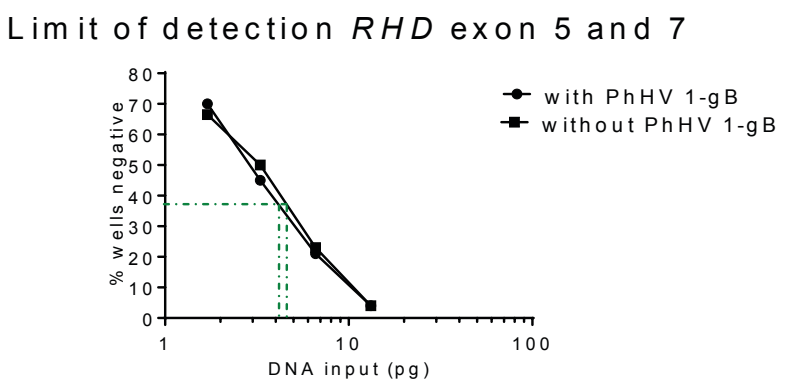

Figure S3 Determination of limit of detection (LOD) for triplex qPCR assay. (A) percentage of positive amplification rate per PCR wells, (B) Mean Ct (±SD) and (C) curve for RHD exon 5 and exon 7. The LOD is determined as the amount where 37% of the PCR experiments performed in 24-48-fold were negative.

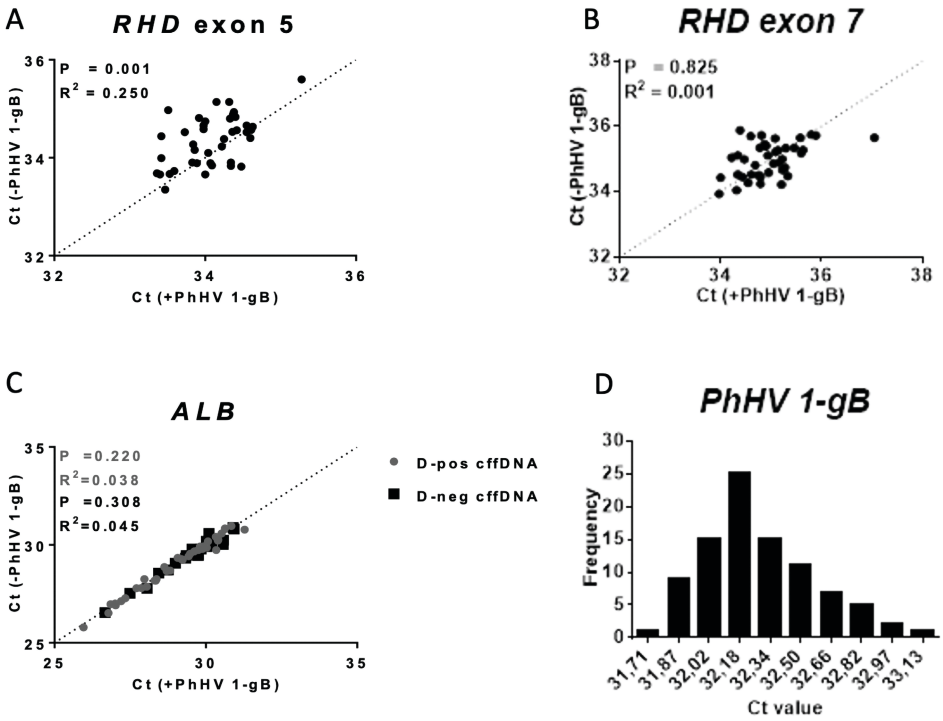

Figure S4 Determination for recovery efficiency of the assay. Amplification of triplex RHD exon 5 (A), exon 7 (B) and PhHV 1-gB (D; histogram frequency), and the single-copy control gene albumin (C) qPCR tested with different plasma pool samples of D-positive cell free DNA in maternal plasma of D-negative pregnant women (●, n = 40) or D-negative cell free foetal DNA in maternal plasma of D-negative pregnant women (■, , *n = 24) spiked with 3335 copies of PhHV 1-gB per PCR well (corresponding to 1000 copies/PCR well) and as control without PhHV 1-gB. Triplex PCRs were performed in triplicate. Albumin PCRs were performed in duplicate.*

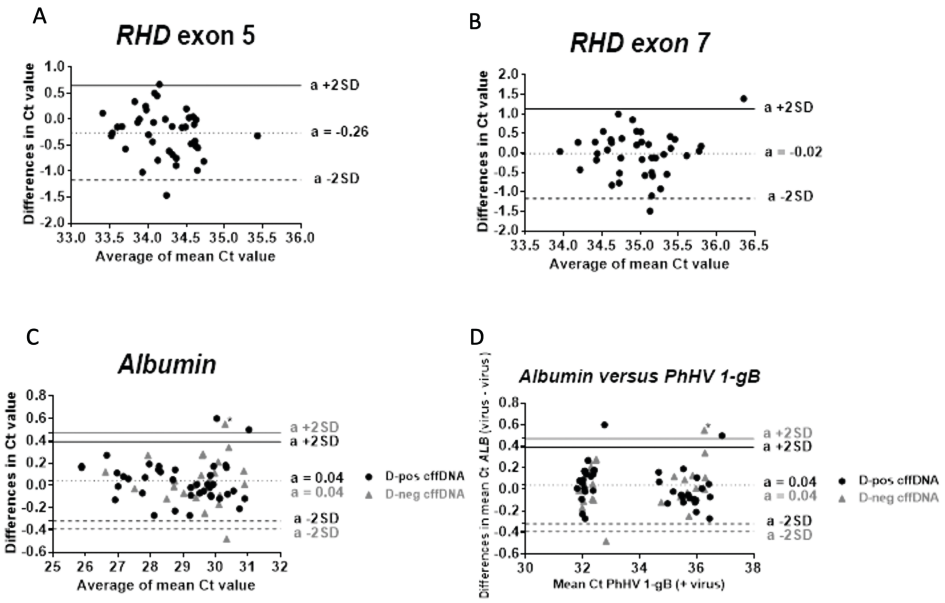

Figure S5 Determination of reproducibility. Differences in mean Ct values against the average of mean Ct values (with PhHV 1-gB compared to the absence of PhHV 1-gB) for triplex RHD exon 5 (A), exon 7 (B) and PhHV 1-gB (D), and the single-copy control gene albumin (C) PCR in D-positive plasma samples and D-negative plasma samples (●, D-positive cell/free foetal DNA in maternal plasma, *n* = 40) and D-negative plasma samples (■, D-negative cell/free foetal DNA in maternal plasma, *n* = 24) spiked with PhHV 1-gB at a fixed concentration of 3335 copies/ml (corresponding to 1000 copies/PCR well). Triplex RHD exon 5, exon 7 and PhHV 1-gB PCR, and albumin PCR experiments were performed in triplicate and duplicate, respectively. a, is the average of the difference between mean Ct values with PhHV 1-gB compared to the absence of PhHV 1-gB and a±2SD represent the upper and lower limits of the test. *The differences in Ct value was between and outside the ±2SD lines, respectively for PhHV 1-gB and albumin qPCR.

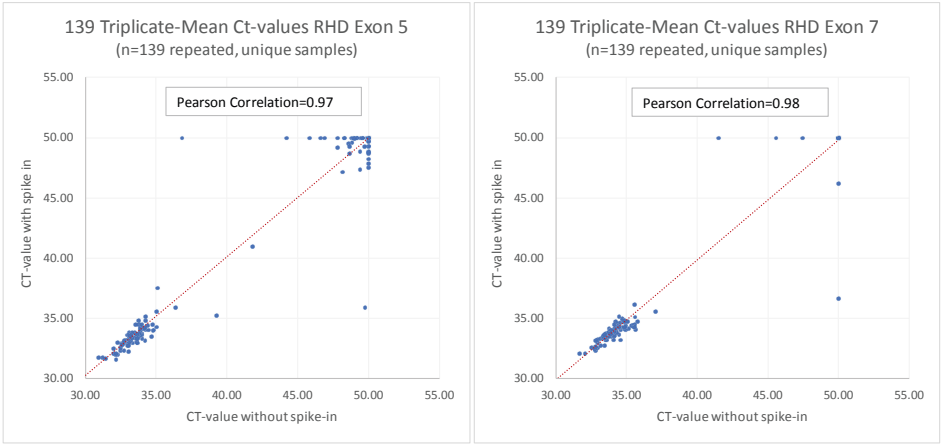

Figure S6 Addition of the spike-in does not impact RHD Ct values. In total 139 samples were tested divided over three runs with or without the spike-in control.

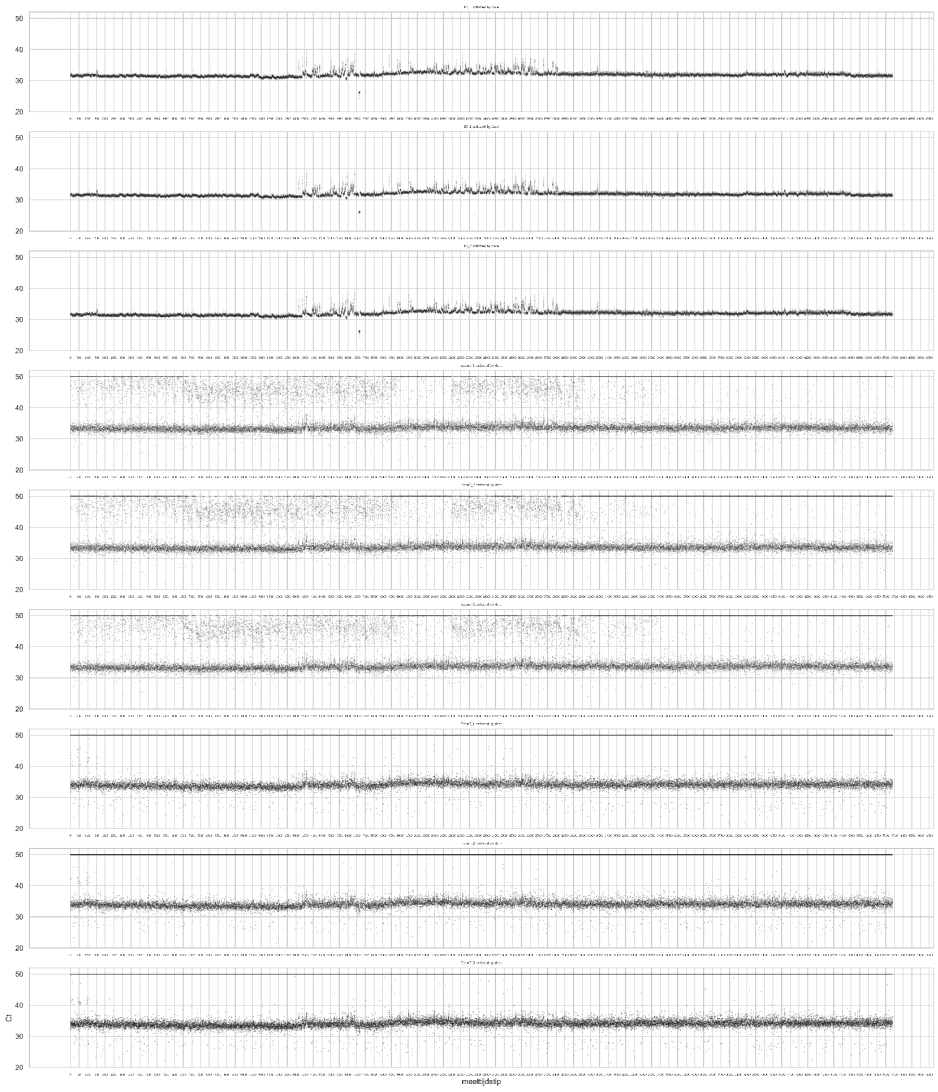

Figure S7 Longitudinal Ct values (single wells) during clinical testing of N = 47,391 between 15feb2022 and 16feb2024. Top three panels are Ct values for spike-in control PhHV1, middle three panels are RHD exon 5 and bottom three panels are RHD exon7. For mean Ct values see Figure 2.

References

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