Noninvasive fetal RHD genotyping to guide targeted anti-D prophylaxis–an external quality assessment workshop

The Noninvasive Fetal RHD Genotyping EQA2017 Working Group

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Title

Noninvasive Fetal *RHD* Genotyping to Guide Targeted Anti-D Prophylaxis – An External Quality Assessment Workshop

Running Title

EQA 2017 Workshop

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(Please note that reference 13 and 14 are not identical)

Abstract

Background and Objectives:

Fetal *RHD* genotyping of cell-free fetal DNA from RhD negative pregnant women can be used to guide targeted antenatal and postnatal anti-D prophylaxis for the prevention of RhD immunization. To assure the quality of clinical testing, we conducted an external quality assessment workshop with the participation of 28 laboratories.

Materials and Methods: Aliquots of pooled maternal plasma were sent to each laboratory. One sample was positive, and the second sample was negative for fetal *RHD*, verified by pre-workshop testing using quantitative real-time PCR (qPCR) analysis of *RHD* exons 4, 5, 7, and 10. Plasma samples were shipped at room temperature. A reporting scheme was supplied for data collection, including questions regarding the methodological setup, results, and clinical recommendations. Different methodological approaches were used, all employing qPCR with a total of eight different combinations of *RHD* exon targets. The samples were tested blindly.

Results: Fetal *RHD* genotyping was performed with no false-negative and no false-positive results. One inconclusive result was reported for the *RHD* positive sample, and four inconclusive results were reported for the *RHD* negative sample. All clinical conclusions were satisfactory.

Conclusion: This external quality assessment workshop demonstrates that despite the different approaches taken to perform the clinical assays, fetal *RHD* genotyping is a reliable laboratory assay to guide targeted use of Rh prophylaxis in a clinical setting.

Key words

Fetal *RHD* genotyping, cell-free fetal DNA, external quality assessment, Rh prophylaxis

Introduction

Alloimmunization against the D antigen in RhD negative pregnant women is preventable using anti-D immune prophylaxis [1,2]. A combination of antenatal and postnatal anti-D administration has dramatically reduced alloimmunization of RhD negative women from approximately 16% to 0.3%, thus decreasing the risk of hemolytic disease of the fetus and newborn [1–4].

Analysis of cell-free fetal DNA (cffDNA) circulating in maternal plasma facilitates noninvasive prenatal testing (NIPT) of the fetal *RHD* genotype during pregnancy [5,6]. Knowledge of the fetal RhD type allows targeted use of antenatal prophylaxis [7], avoiding unnecessary treatment of RhD negative women carrying an RhD negative fetus. These women are at no risk of immunization, and targeted use saves valuable and limited anti-D immunoglobulin [7,8].

Routine fetal *RHD* genotyping to guide targeted prophylaxis using cffDNA has been introduced as a national service in Denmark [9,10], the Netherlands [11], Finland [12], and Norway [13] with several other countries offering the service regionally [14,15]. Other countries, including the UK, are in the process of national implementation [14,15]. As a consequence of the high performance of noninvasive fetal *RHD* genotyping, cord blood typing has been discontinued in Denmark, the Netherlands, and Finland [10,11,16]. Without confirmatory testing of the newborn RhD type, noninvasive fetal *RHD* genotyping is no longer verified, and quality control for the assay becomes increasingly important. Additionally, due to the very low levels of cffDNA present in the maternal plasma, quality assessment is an essential part of safeguarding assay reliability for clinical application of cffDNA testing. Finally, all laboratories (labs) require annual external testing as part of their quality control program. One difficulty has been obtaining sufficient sample volumes to send out to many participants.

Here, we report the results from an external quality assessment workshop, EQA 2017, in which 28 labs participated in testing two pooled plasma samples from RhD negative pregnant women.

Materials and methods

Preparation of plasma pools and sample distribution

Blood was drawn from healthy RhD negative pregnant women at gestational week 25 with informed consent during routine visits to the general practitioner. Blood was collected in 6-mL EDTA tubes and sent to the Department of Clinical Immunology, Copenhagen University Hospital, Denmark, for routine fetal *RHD* genotyping, arriving 2–7 days following venipuncture. The storage and shipment conditions prior to arriving at the laboratory were unknown. However, approximately one third of the blood samples were packed in a Thermo Scientific Nunc Transport Container (Thermo Fisher Scientific Inc., Waltham, MA, USA) and shipped by mail in a padded envelope. Approximately two thirds of the blood samples were transported by car in Smartstore™ Classic 10 (34x25x16 cm) plastic storage boxes with lid (Hammarplast Medical AB, Lidköping, Sweden), where the samples were placed in a foam rack. Upon arrival, plasma was separated by centrifugation at 1,700 x g for 10 minutes. Following routine clinical testing, surplus plasma from 100 *RHD* negative women with *RHD* negative fetuses was pooled to form Pool 1. Surplus plasma from 120 *RHD* negative women with *RHD* positive fetuses was pooled to form Pool 2. Only plasma from samples with a maximum of 4 days transport were used. Surplus plasma was stored at -25° C until pooling. Additional blood group testing of these two pools was approved by the Scientific-Ethical Committees for Copenhagen and Frederiksberg (KF 01283691), which waived the need for written consent. Once the pooling procedure was complete, the individual contributors to the pool could no longer be identified. Both pools were divided into 2-mL aliquots and stored at -25° C until shipping or analysis. Cell-free DNA (cfDNA) was extracted from two aliquots each of Pool 1 and Pool 2 for pre-workshop verification in the lab in Copenhagen. The cfDNA extract was tested for the presence of *RHD* exons 4, 5, 7, and 10, as well as the housekeeping gene *GAPDH*, by quantitative real-time PCR (qPCR) as previously described [10]. Pool 2 was confirmed *RHD* positive with exons 4, 5, 7, and 10, and Pool 1 was confirmed to be *RHD* negative using amplification of the same exons; both pools were positive for *GAPDH* (details presented in Supplementary Information). Fetal and total DNA was quantified as described previously [17] (Supplementary Information). To test the stability of cfDNA in plasma kept at room temperature, plasma aliquots were left at room

temperature for 0 days (n=3), 7 days (n=4), 14 days (n=4), and 21 days (n=4) and subsequently tested for *RHD* exons 7 and 10 and *GAPDH*. The level of cffDNA remained sufficiently high for detection even at the 21-day time point (see Figure 1). A set of Pool 1 and Pool 2 aliquots were then packed directly from storage and sent at room temperature, without temperature control, to 28 external participating labs from the following countries: Argentina, Australia, Denmark, England, Finland, Germany, Iceland, Ireland, the Netherlands, Norway, Poland, Scotland, Singapore, Slovenia, Spain, Sweden, and Switzerland. Participating labs were recruited via network, via information on an ISBT congress poster, and via invitation in the EQA report from 2016; participation was open to everyone. A reporting scheme was supplied for data collection, including questions regarding the method setup, the results, and the clinical recommendations. The samples were tested blindly.

Calculation of Plasma-Equivalents per PCR

The plasma-equivalent per PCR (φ) which indicates the plasma volume actually tested in the PCR [18,19] was calculated as follows:

φ = plasma volume used for DNA extraction x DNA template volume / elution volume

Calculation of Limit of Detection (LOD)

The plasma-equivalent per PCR and the interpretation criteria affects the sensitivity or the LOD [19]. The LOD is defined as the lowest concentration at which 95% of positive samples are detected. A simple approximation formula was used to estimate a theoretical 95% LOD:

LOD (copies per mL) = $3x/n\varphi$

where x is the minimum positive PCR reactions out of a total of n PCR reactions, and φ is the plasma-equivalent per PCR (mL) [19] (see detailed description in Supplementary Information).

Statistics

For comparison of DNA copies in Figure 1, we used Kruskal-Wallis Test with Dunn's Multiple Comparison Test, using GraphPad Prism 5. A p-value below 0.05 was considered significant.

Results

Twenty-eight labs participated in the workshop, of which four labs reported data from two methods; thus, a total of 32 assessments of Pool 1 and 2 were reported. All labs used qPCR for DNA analysis. However, methodologies varied, with differences in protocols for plasma centrifugation, extraction methods (both automated and manual), and in the number and combinations of *RHD* exons used for testing. A summary of the methods used for processing and testing the plasma samples is presented in Table 1. Reporting criteria are detailed in Supplementary Table S1.

Pool 1: RHD negative sample

Twenty-three labs (28 of 32 assessments) correctly reported Pool 1 as *RHD* negative. No false-positive results were observed. Four inconclusive results were reported from the testing of Pool 1. One inconclusive result was due to amplification of exon 10, albeit with a high threshold cycle value (Ct-value); exons 4 and 7 were negative. A second inconclusive result had high Ct-values for exon 5 in 3/8 PCR replicates and was negative for exon 7 in 7/8 replicates. The two remaining inconclusive results were positive for exon 5 and negative for exon 7. In one lab, the exon 5 result was positive with Ct-values equal to those from the positive Pool 2; in the other lab the exon 5 Ct-values were high, although positive in 3/3 replicates. In the latter case, the inconclusive results remained inconclusive in a total of three repeat tests (the lab mentioned that they had initially decided on rather strict criteria for a negative result but will evaluate their criteria as they progress with their routine testing). The clinical conclusions for the inconclusive results were satisfactory, all requesting a new sample and recommending prophylaxis until further analysis.

Pool 2: RHD positive sample

Twenty-seven of 28 participating labs (31 of 32 assessments) found Pool 2 to be *RHD* positive and gave appropriate clinical recommendations for anti-D prophylaxis. No false-negative results were observed. One lab reported an inconclusive result, followed by a request for a new sample. This single inconclusive result had 2/3 positive PCR reactions with high PCR Ct-values, of which only one was positive according to their criteria of Ct of 45 or below. Importantly, the lab noted that the total DNA level was too high according to their algorithm, and in a clinical setting they would have rejected the sample.

A summary of the EQA outcomes is presented in Table 2.

Levels of DNA present in the sample

A subgroup of labs estimated the levels of DNA in their samples. Thirteen labs quantified the number of genome equivalents (geq) of total DNA in their samples or reported DNA levels as ng per µL. The mean estimate of total cfDNA was 26,600 geq/mL for Pool 1 (range: 13,850–34,900 geq/mL) and 19,250 geq/mL for Pool 2 (range: 12,400–30,000 geq/mL). Pre-workshop quantification was 19,800 geq/mL for Pool 1 and 23,300 geq/mL for Pool 2. Seven labs reported estimated levels of cffDNA in Pool 2 with a median of 32 geq/mL (range: 11–305 geq/mL). Pre-workshop quantification was 80 geq/mL. Based on these reports, the overall fetal fraction was 0.06–1.02%. Pre-workshop fetal fraction was estimated at 0.34%.

The plasma-equivalent per PCR could be calculated for all participants (see Supplementary Table S1 for details). Most labs had high plasma-equivalents (Table 2), with 12 labs in the range of 0.1–0.2 mL, and 5 labs in the range of 0.2–0.4 mL. LODs could be calculated for 20 labs and demonstrated generally high sensitivity (Table 2).

Discussion

This report demonstrates the widespread use of accurate fetal *RHD* genotyping as a routine test service to guide targeted prophylaxis. The aim was to provide material for external quality assessment for labs requiring such for their accreditation programs in clinical routine testing. The workshop was designed to lead to successful results, rather than providing difficult, rare, or borderline samples; no *RHD* variants were tested.

We observed high test accuracy with no false-negative results, which is the primary objective when preventing alloimmunization. High sensitivity was expected because the samples were from gestational week 25, when cffDNA is usually readily detected [7]. All results were handled satisfactorily in the context of a clinical setting, with a correct recommendation in every case. Compared with our previous workshop, which included a false-positive result, the overall accuracy was increased [20]. There were several possible causes (discussed further below) for the five inconclusive results reported. This relatively high number would not have been acceptable in a validation study but in an external quality assessment workshop, it reflects the use of various methodologies, experience, and perhaps the high background of maternal DNA.

From previous experience, we knew that high levels of total DNA might affect some assays, leading to inconclusive results [20]. It is possible that high levels of total cfDNA interfered with

cffDNA detection, causing non-specific amplification. This may affect assays that have been optimized at much lower levels of cfDNA; in the case of the inconclusive result for Pool 2, samples tested by the lab are typically obtained from gestational week 10–12, when the levels of cfDNA are lower. Additionally, some labs have clear restrictions concerning the maximum level of cfDNA permissible in their protocols to ensure reliable cffDNA detection, for example, using a maximum of 2 ng cfDNA per PCR or requiring the Ct-value for total DNA to be above 29. The lab reporting the single inconclusive result for Pool 2 had similar restrictions in their algorithm and would have rejected the sample in a clinical setting – exemplifying the value of such criteria.

The exon 5 assay accounted for three of the four inconclusive cases for Pool 1 and might be vulnerable to high cfDNA background. One lab (reporting an exon 5 inconclusive result) mentioned that such inconclusive results were never seen in their routine clinical testing.
In some cases, the cause of inconclusive results might have been the criteria for deciding when a PCR reaction is positive. One lab had similar high Ct-values for exon 5 as observed for some of the inconclusive results, but they were classified as negative reactions according to their algorithm. Adjustment of classification criteria may help prevent future discrepant results. One sample, which repeatedly gave inconclusive results, may have been subject to early contamination.

We only used blood samples received after a maximum of 4 days transportation to reduce the risk of high levels of background maternal genomic DNA from cell lysis. Consequently, the level of background DNA was reduced to approximately 25% of the level observed in the former workshop [20]. Still, the fetal fraction was below 1 %. We will seek to further minimize the level of background DNA in future workshops.

We found that both maternal and fetal plasma DNA levels were stable at room temperature for up to 21 days. This observation, and findings from the literature [21], led us to send the plasma samples without any temperature control. Although it is possible that transportation may have some effect on the levels of cfDNA in the plasma samples, it is unlikely, since following centrifugation, there are few maternal cells remaining in the plasma samples. The estimated level of total DNA before shipment was within the range of the levels estimated by

the labs after transportation, supporting the notion that there is minimal effect of shipping on plasma cfDNA levels, once centrifuged.

The estimates of both total and fetal DNA levels varied between labs. This was to be expected, as differences in methodology may cause different results and since the precision of qPCR-based quantification of low-level DNA is subject to variation. Such variation is also evident in Figure 1, with the cffDNA estimates in the same sample, using the same methodology, ranging from approximately 50 to 100 copies per mL. Nevertheless, quantification measurements can reveal important information about the analysis. Comparing the LOD of the analysis with an estimated average DNA level of the clinical samples that are routinely tested enables a lab to evaluate the robustness of their analysis.

The median LOD of 15.9 geq/mL demonstrates the sensitivity of the methods for detection of the, often, sparse amounts of cffDNA. The LOD is a theoretical measurement, and so an assay with excellent performance can still have a high LOD. However, in general, the LOD is indicative of the assay sensitivity, and can be used to evaluate assays or adjust the assay setup if necessary.

When detecting low-level DNA, such as cffDNA, the risk of false-negative results is present. Using a control assay for the presence of cffDNA has been debated in the field [22] but it is generally accepted that such a control assay is not necessary for noninvasive fetal *RHD* genotyping due to the high sensitivities reported [7]. To date, no optimal control for a screening assay has been identified. Three labs used a universal cffDNA control, demonstrating the presence of cffDNA in their *RHD* negative results. Furthermore, the assessment of total DNA also works as a control for the DNA extraction. Five labs did not use a control for the presence of total DNA.

In addition to the predominantly in-house assays developed by the participating labs, two commercial assays were also included: Nonacus (Nonacus Ltd, Birmingham, England) represented by themselves and one lab applying the FetoGnost Kit RhD and FetoGnost Kit Control (Ingenetix GmbH, Vienna, Austria). Both kits had concordant results.

This workshop provided test material to meet the demand of an annual external quality assessment testing for fetal *RHD* genotyping. Participating labs used different methods, including different combinations of exon targets. Very few discrepancies were observed, indicating that accurate results are achievable despite the differences in method used, and despite testing samples with relatively high background of maternal DNA. We used identical samples from a pool such that each lab received the same sample which made inter-laboratory comparison possible. An EQA participation certificate was provided upon request.

One important limitation is that the relatively high background DNA of the samples may not optimally reflect the clinical routine testing, and as such stresses some of the assays. There is also variability in the gestational age that the participating laboratories offer diagnostic testing and thus the samples used in this scheme will also not reflect the routine samples received by all centres. It is therefore emphasized that the individual performances, especially with the discrepant results, do not directly reflect the performance of the same assays in their respective clinical settings.

This study did not include testing of samples with *RHD* variants. Although the presence of *RHD* variants (maternal or fetal) may present challenges and complicate the detection and interpretation of fetal *RHD* testing, from a practical perspective, it was not possible to collect enough material for such a large number of participating laboratories. Opportunities for future inclusion of *RHD* variant samples will be explored.

Conclusions

The data obtained by the EQA 2017 workshop demonstrate the widespread application of noninvasive fetal *RHD* genotyping as a reliable laboratory assay to guide targeted use of Rh prophylaxis. As an example of precision medicine, targeted prophylaxis helps avoiding unnecessary treatment in pregnant women and saves valuable anti-D immunoglobulin. This approach can also potentially decrease the workload of regional and local immunohematology laboratories by obviating the need to test cord blood for RhD status, as has now become standard in Denmark, the Netherlands, and Finland.

External quality assessment is very important for maintaining the quality of clinical analyses. We will continue to improve this EQA aiming for the most optimal quality assessment of antenatal *RHD* genotyping. We intend to continue to provide a regular EQA scheme open for participation by any laboratories engaged in noninvasive fetal *RHD* genotyping.

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Authorship contribution

FBC designed the study. FBC and ANB drafted the manuscript. FBC, ANB, and all study contributors from the Noninvasive Fetal *RHD* Genotyping EQA2017 Working Group contributed with data analysis, data interpretation, manuscript revision, and approved the final version.

Disclosure of conflict of interests

Dr. Michael Parks and Dr. Lee Silcock are employees at Nonacus Ltd, Birmingham, England.

References

1. de Haas M, Finning K, Massey E, et al.: Anti-D prophylaxis: past, present and future. *Transfus Med* 2014; **24**:1–7.

2. de Haas M, Thurik FF, Koelewijn JM, et al.: Haemolytic disease of the fetus and newborn. *Vox Sang* 2015; **109**:99–113.

3. Turner RM, Lloyd-Jones M, Anumba DO, et al.: Routine antenatal anti-D prophylaxis in women who are Rh(D) negative: meta-analyses adjusted for differences in study design and quality. *PLoS One* 2012; **7**:e30711.

4. Saramago P, Yang H, Llewellyn A, et al.: High-throughput non-invasive prenatal testing for fetal rhesus D status in RhD-negative women not known to be sensitised to the RhD antigen: a systematic review and economic evaluation. *Health Technol Assess* 2018; **22**: 1–172.

5. Lo YMD, Hjelm NM, Fidler C, et al.: Prenatal Diagnosis of Fetal RhD Status by Molecular Analysis of Maternal Plasma. *N Engl J Med* 1998; **339**:1734–1738.

6. Faas BHW, Beuling EA, Christiaens GCML, et al.: Detection of fetal *RHD*-specific sequences in maternal plasma. *The Lancet* 1998; **352**:1196.

7. van der Schoot CE, de Haas M, Clausen FB: Genotyping to prevent Rh disease: has the time come? *Curr Opin Hematol* 2017; **24**:544–550.

8. Kent J, Farrell A-M, Soothill P: Routine administration of Anti-D: the ethical case for offering pregnant women fetal *RHD* genotyping and a review of policy and practice. *BMC Pregnancy and Childbirth* 2014; **14(**87):1–4.

9. Clausen FB, Christiansen M, Steffensen R, et al.: Report of the first nationally implemented clinical routine screening for fetal *RHD* in D- pregnant women to ascertain the requirement for antenatal RhD prophylaxis. *Transfusion* 2012; **52**:752–758.

10. Clausen FB, Steffensen R, Christiansen M, et al.: Routine noninvasive prenatal screening for fetal *RHD* in plasma of RhD-negative pregnant women-2 years of screening experience from Denmark. *Prenat Diagn* 2014; **34**:1000–1005.

11. de Haas M, Thurik FF, van der Ploeg CP, et al.: Sensitivity of fetal *RHD* screening for safe guidance of targeted anti-D immunoglobulin prophylaxis: prospective cohort study of a nationwide programme in the Netherlands. *BMJ* 2016; **355**:i5789:1–8.

12. Haimila K, Sulin K, Kuosmanen M, et al.: Targeted antenatal anti-D prophylaxis program for RhD-negative pregnant women - outcome of the first two years of a national program in Finland. *Acta Obstet Gynecol Scand* 2017; **96**:1228–1233.

13. Sørensen K, Kjeldsen-Kragh J, Husby H, et al.: Determination of fetal *RHD* type in plasma of RhD negative pregnant women. *Scand J Clin Lab Invest* 2018 Jun 5:1–6. [Epub ahead of print] PubMed PMID: 29869532.

14. Daniels G, Finning K, Lozano M, et al.: Vox Sanguinis International Forum on application of fetal blood grouping: summary. *Vox Sang* 2018; **113**:198–201.

15. Daniels G, Finning K, Lozano M, et al.: Vox Sanguinis International Forum on application of fetal blood grouping. *Vox Sang* 2018; **113**:e26–e35.

16. Kuosmanen M, Toivonen S, Haimila K, et al. Non-Invasive Fetal RHD Screening allows discontinuation of Newborn RhD Testing. 35th International Congress of the ISBT, Toronto, Canada, June 2–6, 2018. *Vox Sang* 2018; **113** (Suppl. 1): 39–40.

17. Clausen FB, Jakobsen TR, Rieneck K, et al.: Pre-analytical conditions in non-invasive prenatal testing of cell-free fetal *RHD*. *PLoS One* 2013; **8**:e76990.

18. Legler TJ, Müller SP, Haverkamp A, et al.: Prenatal RhD Testing: A Review of Studies Published from 2006 to 2008. *Transfus Med Hemother* 2009; **36**:189–198.

19. Clausen FB, Urhammer E, Rieneck K, et al.: How to evaluate PCR assays for the detection of low-level DNA. *APMIS* 2015; **123**:731–739.

20. Clausen FB, Barrett AN, Krog GR, et al.: Non-invasive foetal RhD genotyping to guide anti-D prophylaxis: an external quality assurance workshop. *Blood Transfus* 2018; **16**:359–362.

21. Barrett AN, Thadani HA, Laureano-Asibal C, et al.: Stability of cell-free DNA from maternal plasma isolated following a single centrifugation step. *Prenat Diagn* 2014; **34**:1283–1288.

22. Scheffer PG, de Haas M, van der Schoot CE: The controversy about controls for fetal blood group genotyping by cell-free fetal DNA in maternal plasma. *Curr Opin Hematol* 2011; **18**:467–473.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Information on pre-workshop verification of Pools 1 and 2, DNA quantification, and calculation of Limit of Detection (LOD); Supplementary Figure S1: Universal Standard Curve; and Supplementary Table S1: Reporting Criteria for Each Lab

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Figure legends

Figure 1. Plasma aliquots of Pool 2 tested for fetal *RHD* (**a**) and total *GAPDH* (**b**) after storage for 0 to 21 days at room temperature (RT). Results are shown for all PCR reactions. There was no statistically significant difference between the number of copies at the different time points. (**a**) p=0.883 (Kruskal-Wallis Test); no statistically significant differences between groups (Dunn's Multiple Comparison Test), GraphPad Prism 5. (**b**) p=0.082 (Kruskal-Wallis Test); no statistically significant differences between groups (Dunn's Multiple Comparison Test), GraphPad Prism 5. Horizontal bars represent mean; in (**a**) the SEM is also indicated.

Table 1. Summary of methodology for the participating labs (n=32)

Method		**n**
Centrifugation (low speed)	10 min at 1,400–4,300 x *g* (median 1,975 x *g*)	16
	20 min at 2,400 x *g*	1
	5 min at 7,000 x *g*	1
	2nd centrifugation	
	10 min at 9,390 x *g*	1
	10 min at 20,000 x *g*	1
Centrifugation (high speed)	10–20 min at 12,000–20,000 x *g* (median 15,000 x *g)*	6
DNA Extraction	Automated	22
	Manual	9
	(No extraction; direct testing)	1
RHD targets	Exons 5 & 7	15

	Exons 5, 7 & 10	4
	Exons 5 & 10	4
	Exons 7 & 10	4
	Exon 4	2
	Exons 4, 5 & 10	1
	Exons 4, 7 & 10	1
	Exons 4, 5, 7 & 10	1
Total cfDNA targets	*CCR5*	9
	GAPDH	7
	Albumin	5
	β-globin	3
	SOD	1
	EIF2C1	1
	Unspecified maternal marker	1
	None	5
Fetal Marker	*SRY*	7
	RASSF1A/ B-actin	1
	Ins-del polymorphisms	1
	Unspecified fetal marker	1
	None	23

Table 2. Summary of the EQA outcomes

		N (%)
Classification	Pool 1 correctly classified	28 (87.5)
	Pool 1 inconclusive	4 (12.5)
	Pool 2 correctly classified	31 (96.9)
	Pool 2 inconclusive	1 (3.1)
Plasma equivalent per PCR	Median 0.133 mL/PCR (range: 0.033–0.4 mL/PCR)	
Limit of detection	Median 15.9 geq/mL (range 5–76.8 geq/mL)