Clinical Chemistry 61:11
1399–1407 (2015)

Fetal Sex and *RHD* Genotyping with Digital PCR Demonstrates Greater Sensitivity than Real-time PCR

Kelly A. Sillence,[1] Llinos A. Roberts,[2] Heidi J. Hollands,[2] Hannah P. Thompson,[1] Michele Kiernan,[1] Tracey E. Madgett,[1] C. Ross Welch,[2] and Neil D. Avent[1*]

BACKGROUND: Noninvasive genotyping of fetal *RHD* (Rh blood group, D antigen) can prevent the unnecessary administration of prophylactic anti-D to women carrying *RHD*-negative fetuses. We evaluated laboratory methods for such genotyping.

METHODS: Blood samples were collected in EDTA tubes and Streck® Cell-Free DNA™ blood collection tubes (Streck BCTs) from *RHD*-negative women (n = 46). Using Y-specific and *RHD*-specific targets, we investigated variation in the cell-free fetal DNA (cffDNA) fraction and determined the sensitivity achieved for optimal and suboptimal samples with a novel Droplet Digital™ PCR (ddPCR) platform compared with real-time quantitative PCR (qPCR).

RESULTS: The cffDNA fraction was significantly larger for samples collected in Streck BCTs compared with samples collected in EDTA tubes ($P < 0.001$). In samples expressing optimal cffDNA fractions (≥4%), both qPCR and digital PCR (dPCR) showed 100% sensitivity for the *TSPY1* (testis-specific protein, Y-linked 1) and *RHD7* (*RHD* exon 7) assays. Although dPCR also had 100% sensitivity for *RHD5* (*RHD* exon 5), qPCR had reduced sensitivity (83%) for this target. For samples expressing suboptimal cffDNA fractions (<2%), dPCR achieved 100% sensitivity for all assays, whereas qPCR achieved 100% sensitivity only for the *TSPY1* (multicopy target) assay.

CONCLUSIONS: qPCR was not found to be an effective tool for *RHD* genotyping in suboptimal samples (<2% cffDNA). However, when testing the same suboptimal samples on the same day by dPCR, 100% sensitivity was achieved for both fetal sex determination and *RHD* genotyping. Use of dPCR for identification of fetal specific markers can reduce the occurrence of false-negative and inconclusive results, particularly when samples express high levels of background maternal cell-free DNA.

Diagnosis of fetal sex, *RHD* (Rh blood group, D antigen)[3] genotype, and chromosomal abnormalities can be achieved only through analysis of fetal DNA. Initially, this could be achieved through invasive procedures such as amniocentesis and chorionic villus sampling, quoted as having a 1% risk of miscarriage *(1)*. Since the discovery of cell-free fetal DNA (cffDNA)[4] in maternal plasma, noninvasive prenatal testing is now a clinical reality *(1–5)*. Fetal sex determination is offered in the clinic for families at risk of X-linked disorders, such as Duchenne muscular dystrophy *(6)*. Determination of fetal sex is especially beneficial in cases of congenital adrenal hyperplasia, to allow therapy to be targeted to female fetuses only *(7)*. Fetal aneuploidy detection requires accurate quantification and presently can only be determined by next-generation sequencing, which is too costly for routine testing *(8)*.

The antigens of the Rh blood group system are coded for by 2 genes, *RHD* and *RHCE* (Rh blood group, CcEe antigens), which are located on chromosome 1 (p34–p36) *(9)*. In white populations, most D-negative phenotypes result from a complete *RHD* deletion *(10)*. For D-negative individuals of African descent, only 18% are a result of *RHD* deletion. Instead, 66% and 15% of D-negative Africans have an inactive *RHD* gene (*RHDΨ*) or a hybrid gene (*RHD-CE-D*^{S} or *r*^{S}), which do not produce any RhD protein *(10, 11)*. Many laboratories currently provide noninvasive fetal *RHD* genotyping for alloimmunized women as routine practice to manage hemolytic disease of the fetus and newborn (HDFN)

[1] School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, UK; [2] Department of Fetal Medicine, Plymouth Hospitals National Health Service Trust, Plymouth, UK.
* Address correspondence to this author at: School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth, UK PL4 8AA. E-mail neil.avent@plymouth.ac.uk.
Received January 28, 2015; accepted August 24, 2015.
Previously published online at DOI: 10.1373/clinchem.2015.239137

[3] Human genes: *RHD,* Rh blood group, D antigen; *RHCE,* Rh blood group, CcEe antigens; *SRY,* sex-determining region Y; *TSPY1,* testis-specific protein, Y-linked 1; *AGO1,* argonaute RISC catalytic component 1 (formerly *EIF2C1*); *TERT,* telomerase reverse transcriptase; *ERV3-1,* endogenous retrovirus group 3, member 1.
[4] Nonstandard abbreviations: cffDNA, cell-free fetal DNA; HDFN, hemolytic disease of the fetus and newborn; qPCR, real-time quantitative PCR; dPCR, digital PCR; BCT, blood collection tube; cfDNA, cell-free DNA; *RHD5*, Rh blood group, D antigen exon 5; *RHD7*, Rh blood group, D antigen exon 7; FAM, carboxyfluorescein; HEX, hexachlorofluorescein; BLAST, Basic Local Alignment Search Tool; gDNA, genomic DNA; ddPCR, droplet digital PCR; Cq, quantification cycle; Ta, annealing temperature; NTC, no-template control; 2D, 2-dimensional.

Table 1. Summary of amplicon location, length, and fluorescent label for each multiplex reaction.

Multiplex reaction	Amplicon	Chromosome	Gene	Exon/intron	Fluorescent reporter dye	Length, bp	Origin
1	Target	Y	*SRY*	Exon	FAM	137	Lo et al. *(22)*
2	Target	Y	*TSPY1*	Exon	FAM	88	In-house
1 and 2	Reference	X	Xp22.3	Intron	HEX	95	Fan et al. *(23)*
3	Target	1	*RHD5*	Exon (5)	FAM	82	Finning et al. *(24)*
4	Target	1	*RHD7*	Exon (7)	FAM	75	Finning et al. *(24)*
3 and 4	Reference	1	*AGO1*	Exon	HEX	81	Fan et al. *(23)*

(4, 12–14). Before 1970, HDFN was a major cause of fetal mortality (46/100 000 births in the UK alone) *(15)*, but since the introduction of routine antenatal anti-D prophylaxis, incidence has decreased nearly 10-fold *(16)*. Currently, all *RHD*-negative women in the West are offered this prophylaxis, which is costly, as it is produced from hyperimmunized male volunteers. Providing a noninvasive test for fetal *RHD* genotyping allows administration to be targeted to *RHD*-negative women who are known to be carrying an *RHD*-positive fetus. This is now routine in the Netherlands and Denmark with real-time quantitative PCR (qPCR) approaches *(4, 17, 18)*. However, recent publications have described the application of digital PCR (dPCR) for the detection of low-level targets with improved precision, resulting in reliable quantification well below the limit of quantification of qPCR *(19, 20)*.

The use of Streck® Cell-Free DNA™ blood collection tubes (BCTs) instead of conventional EDTA tubes has been shown to increase the proportion of cffDNA *(21)*. Streck BCTs contain proprietary cell-preserving reagents, which prevent maternal cell lysis and consequently reduce the amount of maternal cell-free DNA (cfDNA) released into the plasma. In this study, we compared the sensitivity of dPCR and qPCR for the noninvasive determination of fetal sex and *RHD* genotype for samples collected in both Streck BCTs and EDTA tubes. For technical reasons, some samples, despite being collected in the third trimester, expressed suboptimal cffDNA fractions (<2%). However, all samples were included to thoroughly test the capability of the dPCR assay against the current gold standard, qPCR.

Materials and Methods

STUDY PARTICIPANTS

RHD-negative pregnant women (28–30 weeks' gestation), all of whom met inclusion criteria, were recruited at Plymouth Hospitals NHS Trust, Plymouth, UK, with informed consent, from November 2013 to September 2014. Ethics approval was granted by the United Bristol Healthcare and Trust Research and Ethics Committee (13/SW/0148).

SAMPLE PROCESSING

Twenty-two maternal peripheral blood samples were collected in EDTA tubes (5–10 mL total blood volume) and centrifuged at 1600*g* for 10 min at room temperature (samples 1–22). The plasma was carefully removed and transferred to a 15-mL tube. The plasma was then recentrifuged at 16 000*g* for 10 min. All samples were processed within 4 h of collection, and plasma aliquots (1 mL) were stored at −80 °C. RHD^+ and RHD^- human whole blood, collected in EDTA tubes (5 mL total blood volume), was supplied by National Health Service Blood and Transplant (Bristol, UK) as positive and negative controls, respectively. These samples were processed within 48–96 h by following the same double-spin protocol described above.

Twenty-four maternal blood samples collected in Streck BCTs (10–20 mL total blood volume) were centrifuged at 1600*g* for 15 min at room temperature (samples 23–46). Plasma was carefully removed, transferred to a 50-mL tube, and recentrifuged at 2500*g* for 10 min. All samples were processed within 48 h of collection, and plasma aliquots (1 mL) were stored at −80 °C.

DNA EXTRACTION

DNA was extracted from two 1-mL aliquots of plasma with the QIAamp Circulating Nucleic Acid kit (Qiagen) and QIAvac 24 Plus (Qiagen). The extraction process followed the manufacturer's protocol, and each sample was eluted in 60 μL Buffer AVE [RNase-free water containing 0.04% (wt/vol) sodium azide]. No DNase or RNase treatment was used. After DNA extraction, we quantified samples on the Qubit® 2.0 Fluorometer (Life Technologies) with the Qubit dsDNA HS assay kit (Life Technologies). Samples were stored at −20 °C as 60-μL aliquots for ≤4 weeks.

PCR PRIMERS AND PROBES

For both dPCR and qPCR, we tested 4 multiplex reactions: 2 for fetal sex determination and 2 for fetal *RHD* genotyping (Table 1). Primer concentrations (300 to 900 nmol/L) and annealing temperatures (56 °C to 62 °C) were optimized for all multiplex reactions. Fig. 1 shows

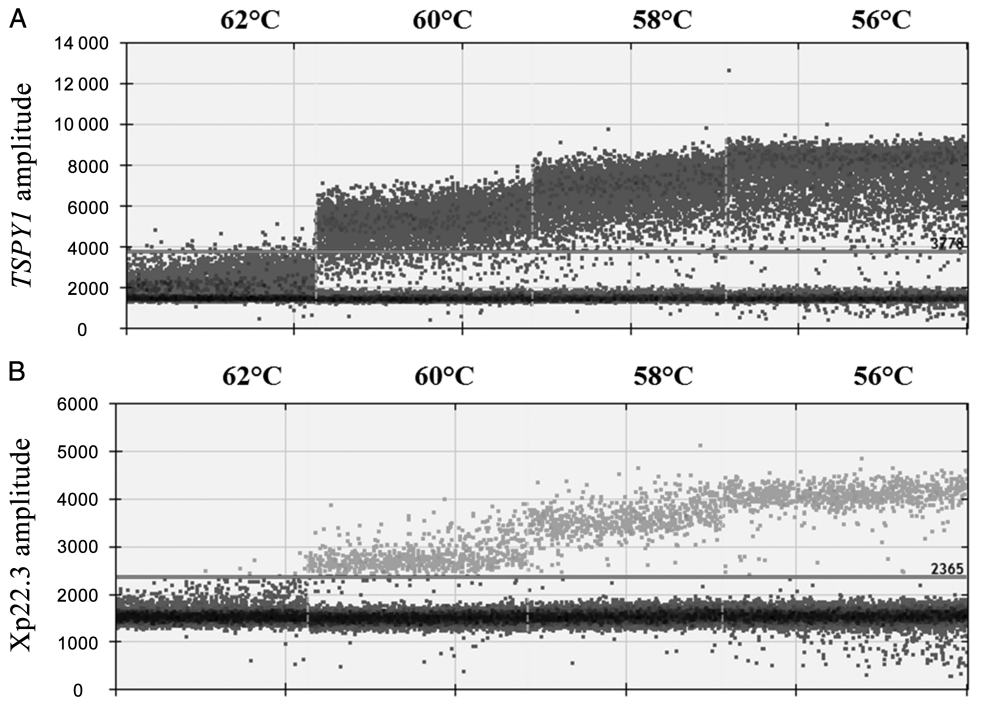

Fig. 1. One-dimensional amplitude plot showing optimization of annealing temperature for *TSPY1*-FAM/Xp22.3-HEX multiplex reaction.

(A), Separation of positive droplets (above threshold line) from negative droplets (below threshold line) for *TSPY1*-FAM amplification (Channel 1). The threshold for *TSPY1* separation was manually set at 4000 amplitude. (B), Separation of positive droplets (above threshold line) from negative droplets (below threshold line) for Xp22.3-FAM amplification. The threshold for Xp22.3 separation was manually set at 2300 amplitude. Results illustrate optimal separation for both targets at 58 °C and 56 °C Ta.

the optimization process for *TSPY1*-FAM/Xp22.3-HEX. We used Xp22.3 oligonucleotides as a reference for 2 Y-specific targets [*SRY* (sex-determining region Y) *(22)* and *TSPY1* (testis-specific protein, Y-linked 1)] for fetal sex determination. *AGO1* (argonaute RISC catalytic component 1; formerly *EIF2C1*) primers were taken from Fan et al. *(23)* and used as a reference for 2 *RHD*-specific targets [*RHD* exon 5 (*RHD5*) and *RHD* exon 7 (*RHD7*)] *(24)* since *AGO1* is also located on chromosome 1. The oligonucleotide sequences (HPLC purified, Eurofins Genomics) and amplicon sizes for all target [carboxyfluorescein (FAM)-labeled] and reference [hexachlorofluorescein (HEX)-labeled] regions are shown in Supplemental File 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue11. Sequences for the Xp22.3 reverse primer and all *TSPY1* oligonucleotides were designed with online software (http://primer3.sourceforge.net and http://www.idtdna.com/calc/analyzer) and subjected to Basic Local Alignment Search Tool (BLAST) analysis against the National Center for Biotechnology Information GenBank DNA database (accession nos. NC_000024.10 and NC_000023.11).

REAL-TIME qPCR

qPCR reactions were performed in a 20-μL solution containing 1× TaqMan Universal PCR Master Mix (Life Technologies), 300 nmol/L primers, 250 nmol/L probes, and a standard volume of template DNA (5 μL). Sample concentrations are recorded in online Supplemental File 2; because of the low abundance of cffDNA in extracted maternal plasma, the samples were not diluted. Reactions were conducted in duplicate, with positive and negative controls for each assay. After optimization of annealing temperature (Ta), cycling was carried out on a Life Technologies StepOnePlus™ qPCR System under the following conditions: 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 58 °C for 1 min. Fifty cycles were used to ensure amplification of low-copy-number target DNA. We included a standard curve of male genomic DNA (gDNA) (Promega) in triplicate on each plate. We used FAM-labeled fluorescent probes for all target regions (*SRY*, *TSPY1*, *RHD5*, and *RHD7*) and HEX-labeled fluorescent probes for both reference regions (Xp22.3 and *AGO1*) (Table 1; also see online Supplemental File 1).

Table 2. Summary of fetal sex determination and *RHD* genotyping results obtained from both dPCR and qPCR against results recorded after delivery.

		Sex determination					*RHD* determination				
		Male		Female			Positive		Negative		
Platform	Samples	Fetus	Newborn	Fetus	Newborn	Inconclusive	Fetus	Newborn	Fetus	Newborn	Inconclusive
dPCR	46										
EDTA tubes	22	10	10	12	12	0	12	12	9	10	1
Streck BCTs	24	13	13	11	11	0	19	19	5	5	0
qPCR	46										
EDTA tubes	22	10	10	12	12	0	0	12	22	10	0
Streck BCTs	24	13	13	11	11	0	15	19	5	5	4

Digital PCR

dPCR reactions were performed in a 20-μL solution containing 10 μL droplet digital PCR (ddPCR) Supermix for Probes (Bio-Rad), 300 nmol/L primers, and 250 nmol/L probes. Because samples were not diluted after Qubit quantification, we added a standard volume of template DNA (5 μL) with positive and negative controls. All reactions were conducted in duplicate and run on the QX100™ Droplet Generator (Bio-Rad) according to the manufacturer's instructions. By use of an oil emersion approach, the sample was drawn through the cartridge under a vacuum, where approximately 20 000 1-nL droplets were formed. The droplets (40 μL total volume) were then transferred to a 96-well plate and covered with a pierceable foil heat seal on the PX1™ Plate Sealer (Bio-Rad). Cycling was carried out on a C1000 Touch™ Thermal Cycler (Bio-Rad) under optimized conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 58 °C for 1 min, after which a final 98 °C step for 10 min was carried out (as recommended by Bio-Rad). Samples were analyzed immediately on the QX100^{TM} Droplet Reader (Bio-Rad). To ensure uniformity, samples were extracted and tested by qPCR and dPCR on the same day by the same investigator.

DATA ANALYSIS

For qPCR, targets with a mean quantification cycle (Cq) of <45 were recorded as positive, provided no-template controls (NTC) remained negative (Cq >45), to ensure inclusion of low-copy-number targets. Targets that expressed Cq values >45 for both duplicates were recorded as negative. Thresholds were set at 0.05 for all targets (StepOne™ Software v2.3).

We analyzed the raw fluorescent data from the dPCR platform with Bio-Rad QuantaSoft v1.2 software. Once thresholds for each sample had been set manually with the 2-dimensional (2D) amplification plot, positive and negative droplets were determined (Fig. 1; also see online Supplemental File 3). Thresholds were determined when intermediate droplets between 2 clusters did not alter the calculated concentration (Poisson 95% CI) *(20)*. Online Supplemental File 4 shows the calculations used for determining the cffDNA fraction by use of the dPCR results on the basis of the concentration (copies per microliter). All statistical analysis for comparing Streck BCTs vs EDTA tubes for both cffDNA fractions and reference DNA concentration were performed with Mann–Whitney *U* test (SigmaPlot v12.5), and significance was accepted at $P < 0.05$.

CONFIRMATION OF FETAL SEX AND *RHD* STATUS

The accuracy of dPCR and qPCR for the prenatal detection of fetal sex was ascertained at birth (Table 2). Fetal blood group was verified after delivery through the serology of umbilical cord blood samples.

Results

FETAL SEX DETERMINATION

For dPCR, in 100% of cases, the fetal sex predicted by using both Y-specific targets (*TSPY1* and *SRY*) was the same as that determined at birth (Table 2). The Ta gradient was optimized for all targets, and despite Fig. 1 illustrating equal separation at 58 °C and 56 °C, the 2D amplitude plot illustrated better separation at 58 °C (data not shown). In addition, at Ta 58 °C, the *SRY*-FAM/Xp22.3 multiplex reaction produced a ratio closer to 1 than at Ta 56 °C (0.931 and 0.835, respectively). The *SRY* assay was successful only for the male positive control by qPCR. Therefore, fetal sex was ascertained by *TSPY1* only for qPCR (Table 2; also see online Supplemental File 2). The results also illustrated 100% accuracy when only the multiple-copy target gene was considered for qPCR analysis (Table 3). Calibration curves, slopes, *y*-intercepts, R^2 values, and efficiencies for qPCR data are shown in online Supplemental File 5.

Table 3. Results of testing 22 and 24 maternal samples collected in EDTA tubes and Streck BCTs, respectively, with dPCR and qPCR for fetal sex and *RHD* genotyping.

Platform and target gene	Sensitivity, %	False-negative results, % (n)	Specificity, %	False-positive results, % (n)	Accuracy, %[c]
dPCR					
Streck BCTs[a]					
TSPY1	100		100		100
SRY	100		100		100
RHD5	100		100		100
RHD7	100		100		100
EDTA tubes[b]					
TSPY1	100		100		100
SRY	100		100		100
RHD5	100		95.5	4.5 (1)	95.6
RHD7	100		95.5	4.5 (1)	95.6
qPCR					
Streck BCTs[a]					
TSPY1	100		100		100
SRY	50	54.2 (13)	100		45.8
RHD5	83.4	16.6 (4)	100		83.4
RHD7	100		100		100
EDTA tubes[b]					
TSPY1	100		100		100
SRY	0	45.5 (10)	100		54.5
RHD5	0	59.1 (13)	100		40.9
RHD7	0	59.1 (13)	100		40.9

[a] cffDNA in maternal plasma 4%–24%, calculated from dPCR results.
[b] cffDNA in maternal plasma 0.1%–2%, calculated from dPCR results.
[c] Accuracy was calculated as (true positives + true negatives)/(true positives + false positives + false negatives + true negatives).

The cffDNA fraction with both Y-specific and *RHD*-specific targets was calculated on the basis of the concentration (copies per microliter) generated by dPCR for each multiplex reaction (see online Supplemental File 4). The samples collected in Streck BCTs expressed higher cffDNA fractions (4%–24%) and were classed as optimal, whereas the samples collected in EDTA tubes, during the initial stages of sample collection, illustrated lower cffDNA fractions (0.1%–2%) and were classified as suboptimal (Fig. 2A).

FETAL *RHD* GENOTYPING

By dPCR, fetal *RHD* genotype was correctly identified in 100% (24/24) and 95.5% (21/22) of cases for samples collected in Streck BCTs and EDTA tubes, respectively (Table 3). One EDTA-collected sample (sample 12) produced a false-positive result, since serological analysis revealed the fetus to be *RHD*-negative but dPCR showed clear amplification of the *RHD7* target (18 droplets) and minimal amplification of the *RHD5* target (3 droplets) (Fig. 3A). Fig. 3 illustrates the concentrations obtained from both target genes (*RHD5* and *RHD7*) and the reference gene (*AGO1*) for control samples [NTC, RHD^+ control, RHD^- control (Fig. 3, A and B); samples collected in EDTA tubes (Fig. 3A); and samples collected in Streck BCTs (Fig. 3B)]. The results show successful amplification of all 3 targets for the *RHD*-positive control sample and show amplification of only the reference *AGO1* gene for the *RHD*-negative control sample, whereas the NTC sample showed no amplification (Fig. 3). In addition to the false-positive result (1/46, 2%), 31 samples were correctly classified as *RHD* positive (67%) and 14 samples were correctly classified as *RHD* negative (31%) (Fig. 3).

Optimal samples (collected in Streck BCTs), which expressed cffDNA fractions ≥4%, demonstrated accuracies of 100% and 83% on the qPCR platform for the

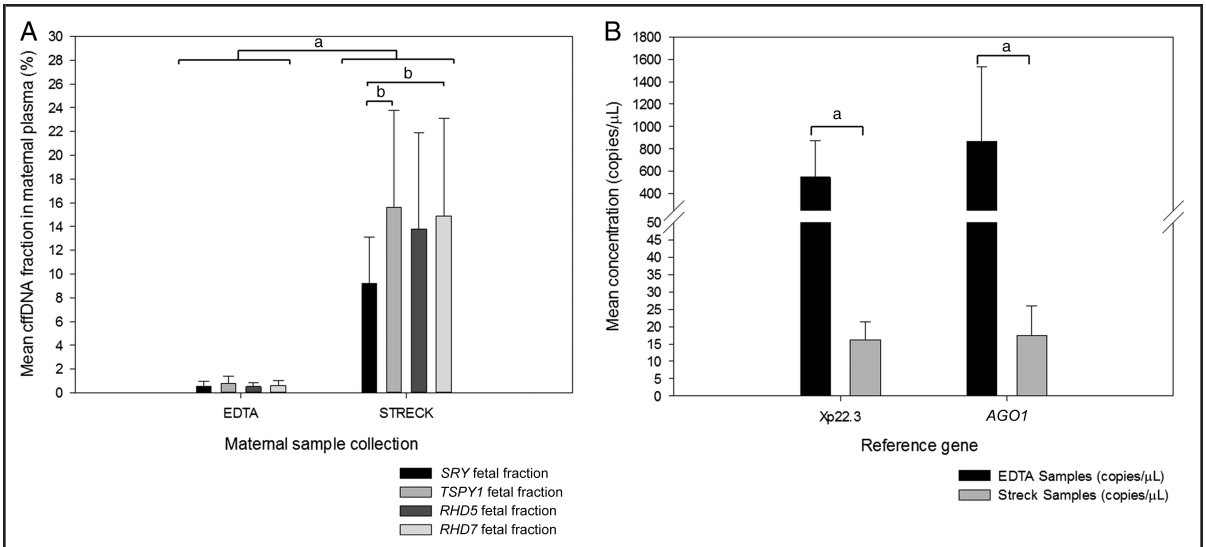

Fig. 2. Comparison between maternal samples collected in EDTA tubes and Streck BCTs.

(A), Mean cffDNA fraction in maternal plasma calculated by each target gene (see online Supplemental File 1). The samples collected in Streck BCTs show a significantly higher mean cffDNA fraction compared with samples collected in EDTA tubes for all 4 target regions ($^aP < 0.001$). The cffDNA fractions on the basis of the *RHD7* and *TSPY1* target genes are significantly larger than the cffDNA fraction determined by the *SRY* target gene ($^bP < 0.01$). (B), Mean concentration of reference gene regions Xp22.3 and *AGO1* for maternal samples collected in EDTA tubes and Streck BCTs. The mean concentrations of both regions were significantly higher in EDTA tube samples than in Streck BCTs ($^bP < 0.001$). There was no significant difference between mean concentrations of Xp22.3 and *AGO1* within each sample collection method.

RHD7 and *RHD5* target assays, respectively. Four samples (16.6%) were classified as inconclusive because qPCR did not detect the *RHD5* target but did show acceptable amplification of the *RHD7* target (<45 Cq) (Table 3). The qPCR platform was unable to detect both *RHD*-specific markers (*RHD7* and *RHD5*) in the suboptimal samples (<2% cffDNA), despite serological and dPCR analysis confirming that 59% (13/22) of these EDTA-collected samples were carrying an *RHD*-positive fetus.

SAMPLE COLLECTION: EDTA VS STRECK BCT

The cffDNA fractions and concentrations of reference targets for blood collection methods with EDTA tubes and Streck BCTs were compared by use of dPCR results. Fig. 2A shows the mean cffDNA fraction in maternal plasma for all 4 target regions (*SRY*, *TSPY1*, *RHD5*, and *RHD7*) for both collection methods. The samples collected in Streck BCTs showed significantly larger mean cffDNA fractions (9%–16%) for all target regions than those collected in EDTA (0.5%–1%) ($P < 0.001$). The mean cffDNA fractions generated by the EDTA-collected samples demonstrated no significant differences between all 4 targets ($P > 1$). However, the cffDNA fraction calculated on the basis of the *SRY* target was significantly smaller than the *TSPY1* and *RHD7* cffDNA fractions ($P < 0.01$).

Although the concentration of each reference gene (Xp22.3 and *AGO1*) (Fig. 2B) is a combination of maternal and fetal cfDNA, it is predominantly maternal in origin (90%–95%). Samples collected in Streck BCTs showed similar mean concentrations for Xp22.3 and *AGO1* reference genes (16.18 and 17.39 copies/μL, respectively; $P > 0.1$) (Fig. 2B). The concentrations of both reference targets (Xp22.3 and *AGO1*) were >40-fold higher for maternal samples collected in EDTA tubes compared with Streck BCTs (mean concentrations 548.04 and 869.25 copies/μL, respectively), suggesting maternal leukocyte degradation (Fig. 2B). The 2D amplification plots (see online Supplemental File 3) also showed a significantly higher number of reference (HEX-labeled) droplets for maternal samples collected in EDTA tubes compared with maternal samples collected in Streck BCTs ($P < 0.001$). The fetal concentration determined from *RHD5* and *RHD7* amplification was similar for samples collected in both EDTA and Streck BCTs ($P > 0.1$): 0.9–4.2 copies/μL and 0.3–3.7 copies/μL, respectively (Fig. 3).

Discussion

Noninvasive fetal *RHD* genotyping by use of qPCR analysis has shown high levels of accuracy for optimal samples (mean 97.4%) and is currently implemented in the Neth-

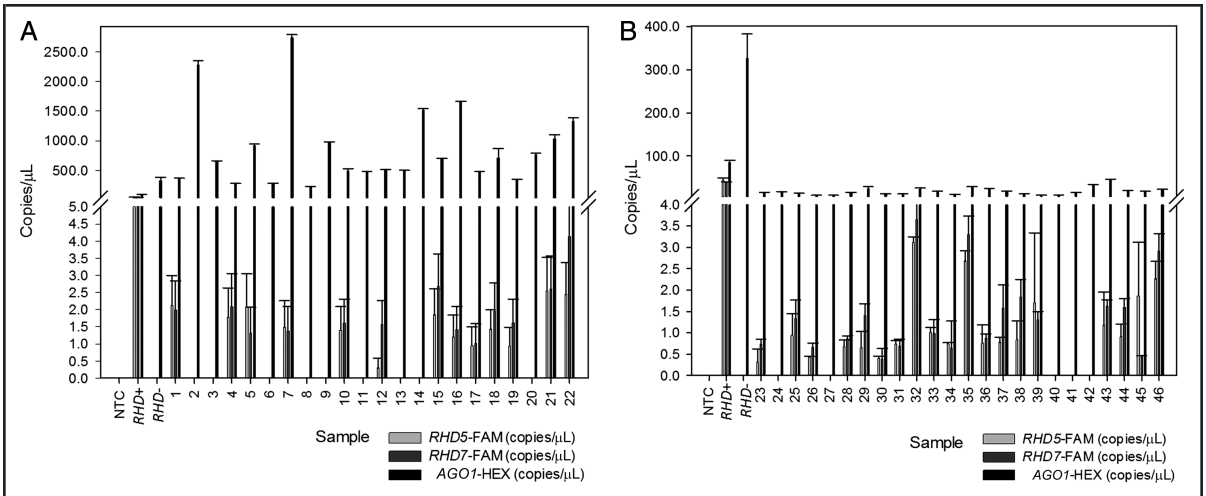

Fig. 3. Fetal *RHD* genotyping results from maternal plasma samples.

The concentration (copies per microliter) (plus SD) was identified for both target regions (*RHD5* and *RHD7*) and the reference region (*AGO1*). The presence or absence of the target regions were used to determine fetal status (RHD^+ or RHD^-, respectively). (A), Maternal samples collected in EDTA tubes (n = 22). (B), Maternal samples collected in Streck BCTs (n = 24). The same controls are represented in both graphs. The control nonmaternal cfDNA *RHD*-positive sample (399X) exhibited ratios of 0.51 and 0.47 for *RHD5*/*AGO1* and *RHD7*/*AGO1*, respectively.

erlands and Denmark for targeted administration of prophylactic anti-D *(4, 10, 11, 17, 18, 25)*. In a recent population-based cohort study, mass-throughput fetal *RHD* genotyping was sufficient from the end of the first trimester with qPCR *(26)*. However, for samples taken at <11 weeks' gestation, 16 of 865 samples (1.8%) were incorrectly classified as *RHD* negative. Fetal *RHD* genotyping was also inconclusive for 393 of 4913 samples tested (8%) *(26)*. Previous studies have identified that low cffDNA fractions can lead to false-negative results by qPCR, limiting the sensitivity of this approach *(27–29)*. The oligonucleotides used in this study for *RHD* genotyping are identical to the sequences used in Finning et al. *(24)*, but for unknown reasons worked less effectively for our qPCR assay. However, our results clearly indicate that for suboptimal samples, the single-copy targets (*SRY, RHD5*, and *RHD7*) were not detectable by qPCR but achieved 100% sensitivity (95% CI) on the dPCR platform.

Because of the gestational ages of these samples, cffDNA is expected to be >5% *(1)*. However, results show maternal DNA degradation for EDTA-collected samples, since the number of positive droplets for references (Xp22.3 and *AGO1*) was significantly higher compared with samples collected in Streck BCTs ($P < 0.001$) (Fig. 2B; also see online Supplemental File 3). These novel dPCR data indicate that qPCR false-negative results were not caused by low absolute cffDNA concentrations, since they are similar to those expressed by optimal samples (Fig. 2B), but are instead a result of low relative concentrations of cffDNA. The assay used is highly specific, and theoretically, nonspecific amplification should not occur, but because *RHD5* and *RHD7* probes have 96.5% and 100% consensus, respectively, with the *RHCE* gene, it is possible that the probes are binding to the abundant maternal *RHCE*, depleting probe availability for fetal-specific *RHD* targets. Nonetheless, when the cffDNA copy number is very low, false-negative results are more likely, particularly for the detection of fetal single nucleotide polymorphisms for rare mutation detection. qPCR is more susceptible to nonspecific amplification of the maternal allele, and dPCR maybe more powerful in the detection of alleles associated with conditions such as β-thalassemia and cystic fibrosis.

The amount of fetal DNA fraction has been shown to increase in positive correlation with time before processing *(30)*. To preserve large cffDNA fractions, it is recommended that samples collected in EDTA tubes should be extracted within 6 h and kept at 4 °C before plasma extraction. Although maternal samples collected in EDTA were processed within 6 h, all transportation of these samples between sites was carried out at room temperature for logistical reasons. The chosen references (Xp22.3 and *AGO1*) were based on assumptions that cfDNA is fragmented equally across the genome, and dPCR analysis showed equal abundance of reference to target loci for nonmaternal cfDNA samples, since a ratio of approximately 1 was expressed (see online Supplemental File 3). This is important, because a previous study has shown unequal representation of reference targets [e.g., *TERT* (telomerase reverse transcriptase) and *ERV3–1*

(endogenous retrovirus group 3, member 1)] in cfDNA compared to genomic DNA *(31)*. In addition, the RHD^+ control used in Fig. 3 shows a ratio of approximately 0.5, illustrating that the control sample is hemizygous for the *RHD* gene, whereas homozygous *RHD* samples tested with the same assay expressed ratios close to 1 (data not shown).

dPCR data were used to determine the cffDNA fraction, since they are expected to demonstrate higher levels of sensitivity and improved accuracy for low template copy numbers *(32)*. Poisson statistics were incorporated to determine the copy number, since some droplets may have contained multiple targets *(33)*. The proportion of fetal target DNA was relatively low [mean number of droplets 12 084, mean number of *RHD* molecules 20.8 and 17.8 for samples from EDTA tubes and Streck BCTs, respectively (data not shown)]. However, increases in the mean number of copies per partition for the references (e.g., *AGO1*), which is shown in EDTA maternal samples [e.g., 0.62 mean copies per partition (sample 15)], result in higher proportions of dual-positive droplets compared to samples collected in Streck BCTs [e.g., 0.023 mean copies per partition (sample 32)] (see online Supplemental File 3). Since the release of the QX100™ ddPCR system in 2012, various studies have been conducted to find out whether its application can enhance or replace current qPCR-based approaches *(19, 20, 33–36)*. Some studies have shown equal sensitivity for dPCR and qPCR, but with improved levels of precision and day-to-day reproducibility with dPCR approaches *(35, 36)*. However, several studies have shown considerable improvements of sensitivity and specificity on the dPCR platform compared with qPCR approaches *(19, 20, 33, 34)*. The current study also illustrates significant improvements in sensitivity for the dPCR platform, particularly for samples expressing low relative proportions of fetal DNA (<2%) (Table 3).

On the basis of the qPCR data, 54% of patients had false-negative results and in a clinical setting would not have received required anti-D, risking alloimmunization and subsequent HDFN. However, dPCR results revealed no false-negative results, and routine administration of this assay would have prevented unnecessary anti-D administration in 31.1% of patients in our study cohort. Previous studies have also reported false-positive or inconclusive results when the fetus expresses D-variants *(4, 20, 24)*. False-positive results do not pose a risk of alloimmunization but result in unnecessary anti-D administration. If applied to a clinical setting, anti-D would have been administered to the 4 women with inconclusive results found with qPCR for samples collected in Streck BCTs, which in these cases was necessary since the fetuses were RHD^+. On the basis of the dPCR data, only 1 woman (2%) would have received anti-D that was not required. The oligonucleotide primers used in this study for the *RHD* targets were as described by Finning et al. *(24)*. These primers should distinguish between *RHD*-positive and *RHDΨ*/DVI (type 1–4) fetal genotypes by amplifying exon 7 but not exon 5 for the variant samples. However, constraints on ethics approval prevented follow-up confirmation of the inconclusive result (sample 37) via analysis of fetal cord DNA to determine the true *RHD* genotype of this fetus. Both dPCR and qPCR will express similar levels of false-positive results owing to D-variants, but our results show that dPCR has the potential to eliminate or reduce the occurrence of false-negative results, especially in cases in which low cffDNA fractions (<2%) are expressed.

In conclusion, this study illustrates that dPCR shows improved accuracy for fetal sex determination and *RHD* genotyping compared with qPCR, particularly for suboptimal samples that express low relative proportions of fetal DNA (<2%). Despite the accuracy of qPCR being relatively high in most large-scale validation studies *(3, 9, 24)*, false-negative results are still present and have been attributed to maternal DNA degradation. Further large-scale studies are now necessary to determine the accuracy of dPCR for fetal *RHD* genotyping, but these results illustrate that dPCR has the potential to provide a safer and more reliable noninvasive diagnostic test for the targeted administration of prophylaxis anti-D.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: N.D. Avent, Grifols AG.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: K.A. Sillence, bursary from Plymouth University.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played a direct role in the design of study, choice of enrolled patients, review and interpretation of data, and preparation or approval of manuscript.

Acknowledgments: Special thanks to all the pregnant women who took part in this study. We also thank all the staff at Plymouth Hospitals NHS Trust for their advice and support throughout sample collection.

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