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7 **Rapid *RHD* zygosity determination using digital PCR**

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15 **Keywords:** Zygosity, digital PCR (dPCR), long-range PCR (LR-PCR), next
16 generation sequencing (NGS), genotype, serology and single nucleotide
17 polymorphisms (SNPs).

18 **List of Abbreviations:** Hemolytic disease of the newborn and fetus (HDFN), real-
19 time PCR (qPCR), digital PCR (dPCR), minutes (mins), double-stranded DNA
20 (dsDNA), droplet digital PCR (ddPCR), *RHD* exon 5 (*RHD5*), *RHD* exon 7 (*RHD7*),
21 High Performance Liquid Chromatography (HPLC), seconds (s), long-range PCR
22 (LR-PCR), next generation sequencing (NGS), Ion sphere particles (ISPs), Ion
23 Personal Genome Machine™ (Ion PGM™), Variant Caller Files (VCF), human

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24 genome 19 (hg19), Integrative Genomics Viewer (IGV), the Single Nucleotide
25 Polymorphism Database (dbSNP), single nucleotide polymorphism (SNP), Browser
26 Extensible Data (BED), cell-free DNA (cfDNA), National Center for Biotechnology
27 (NCBI).

28 **Human Genes:** Reference genes: *AGO1* (argonaute RISC catalytic component 1,
29 HGNC: 3262) (or *AGO1* (eukaryotic translation initiation factor 2C, 1)). Target genes:
30 *RHD* (Rh blood group, D antigen, HGNC: 10009) (or Rhesus blood group, D
31 antigen).

32 **Abstract**

33 **Background:** Paternal zygosity testing is used to determine the hemi- or
34 homozygosity of *RHD* in pregnancies at risk of hemolytic disease of the newborn and
35 fetus (HDFN). Currently, this is achieved using real-time PCR or the RH box PCR,
36 which can be difficult to interpret and unreliable, particularly for black African
37 populations.

38 **Method:** DNA samples extracted from 58 blood donors were analysed using two
39 multiplex reactions for *RHD* specific targets against a reference (*AGO1*) to determine
40 gene dosage using digital PCR. Results were compared to serological data and the
41 correct genotype for two discordant results was determined by long range-PCR, next
42 generation sequencing and conventional Sanger sequencing.

43 **Results:** The results show clear and reliable determination of *RHD* zygosity using
44 digital PCR and revealed that four samples did not match the serologically predicted
45 genotype. Sanger sequencing and long range-PCR followed by next generation
46 sequencing revealed that the correct genotypes for samples 729M and 351D, which
47 were serologically typed as R_1R_2 (DCE/dCE), were R_2r' (DcE/dCe) for 729M and R_1r''
48 (DCE/dcE), R_0r^y (Dce/dCE) or R_zr (DCE/dce) for 351D, in concordance with the
49 digital PCR data.

50 **Conclusion:** Digital PCR provides a highly accurate method to rapidly define blood
51 group zygosity, and has clinical application in the analysis of Rh phenotyped or
52 genotyped samples. The vast majority of current blood group genotyping platforms
53 are not designed to define zygosity, and thus this technique maybe used to define
54 paternal *RH* zygosity in pregnancies at risk of HDFN, and distinguish between homo-
55 and hemizygous *RHD* positive individuals.

56 **Introduction**

57 Of the 36 blood group systems Rh is the most complex at the genetic level and is the
58 major cause of hemolytic disease of the fetus and newborn (HDFN) and a major
59 cause of transfusion reactions during alloimmunization events. The *RH* genes, *RHD*
60 and *RHCE*, are well characterized at the genetic level, with a combination of SNPs in
61 *RHCE* being responsible for the C/c and E/e polymorphisms, whilst gene deletion
62 and hybrid *RHD-RHCE* genes are responsible for D-negative phenotypes, plus
63 SNPs and hybrid genes being responsible for partial and weak D phenotypes (1, 2).
64 All known mutations have been well catalogued and best described in the
65 RhesusBase resource (3).

66 Paternal *RHD* zygosity testing is important for prenatal management of
67 alloimmunized women. Where fathers are homozygous D- there is no risk of HDFN
68 for the current pregnancy or subsequent pregnancies that may follow. Pregnancies
69 to homozygous D+ fathers (with the assumption of paternity) will by definition carry
70 RhD positive fetuses, and can be considered for more focused clinical management.
71 For hemizygous D+ fathers non-invasive prenatal testing (NIPT) is required for a
72 definitive diagnosis.

73 Previously published methods for *RHD* zygosity testing have included real-time PCR
74 (qPCR) assessment of *RHD* gene dosage, assessment of the hybrid *Rhesus box*
75 found in D-negative individuals with the *RHD* gene deletion genotype and allele-
76 specific PCR methods, as well as mass spectrometry-based methods. (2, 4-10).
77 Zygosity testing targeting the hybrid *Rhesus box* found in *RHD*-deletion type cde
78 haplotypes is complicated because of differences in the hybrid box amongst
79 individuals of African descent (5, 11).

80 The incidence of common *RH* haplotypes in Caucasian, African black and Asian
81 populations has been defined serologically. In *RHD* positive individuals the D_{Ce}
82 haplotype is prevalent in Asian (73%) and Caucasian (42%) populations, but in
83 African black populations the D_{ce} haplotype has a higher incidence (59%) (12). *RHD*
84 negative individuals are rarely found in Asian populations (<4%), but the d_{ce}
85 haplotype is frequently found in Caucasian (39%) and African black (20%)
86 populations (12). Rare haplotypes such D_{CE}, d_{Ce}, d_{cE} and D_{ce} are considerably
87 less prevalent with frequencies of 0.24%, 0.98%, 1.19% and 2.57% respectively, in
88 Caucasian populations (12). However, it has been difficult to define the precise
89 population frequencies of the various *RH* haplotypes due to the inability to
90 differentiate between hemi- or homozygous individuals. For example, an individual
91 with the phenotype D_{Ce} would be designated as the most common presumed
92 genotype D_{Ce}/D_{Ce} rather than D_{Ce}/d_{Ce}. Thus presumed genotype, based on
93 probability, is the manner in which donor and patient red cells are labelled. Zygosity
94 determination of the above would define which presumed genotype (D_{Ce}/D_{Ce} or
95 D_{Ce}/d_{Ce}) (two copies of the *RHD* gene versus one copy of the *RHD* gene) is carried
96 by a particular individual.

97 Previously we have applied digital PCR (dPCR) to the analysis of free fetal DNA
98 derived from maternal plasma (13). In this study we have utilized dPCR as a more
99 accurate quantitative PCR method than conventional qPCR to define *RHD* zygosity.
100 We found rare haplotypes in a relatively small cohort of samples and identified that
101 for three samples (plus one weak D sample) their predefined and labelled presumed
102 genotype was indeed incorrect.

103 **Materials and Methods**

104 **Study Participants**

105 Human whole blood samples ($n = 79$) were supplied by the National Health Service
106 Blood and Transplant (NHSBT) (Bristol, UK) (donated with informed consent) and
107 transported to NHS Plymouth Hospitals Trust, Plymouth, UK for collection.

108 **Sample Processing**

109 Samples were processed in two ways. Human whole blood samples ($n = 25$) were
110 collected in EDTA tubes (5-10 mL total blood volume) and centrifuged at 1 600xg for
111 10 minutes (min) at room temperature. The plasma was carefully removed and
112 transferred to a 15 mL tube. The plasma was then re-centrifuged at 16 000xg for 10
113 min. All samples were processed within 48 to 96 hours of collection and plasma
114 aliquots (1 mL) were stored at -80°C .

115 Human whole blood samples ($n = 54$) were collected in EDTA tubes (5-10 mL total
116 blood volume) and centrifuged at 2 500xg for 10 min at room temperature. The buffy
117 coat layer was carefully removed and transferred to a 1.5 mL tube for immediate
118 processing to genomic DNA (gDNA). All blood samples were processed within 48 to
119 96 hours of blood collection.

120 **DNA extraction from plasma**

121 Plasma extractions were performed as non-pregnant controls from maternal plasma
122 experiments (13) and were further utilised in this study. DNA was extracted from two
123 1 mL aliquots of plasma using the QIAamp Circulating Nucleic Acid (CNA) kit
124 (Qiagen, West Sussex, UK) using the QIAvac 24 Plus (Qiagen). The extraction
125 process was as the manufacturer's protocol and each sample was eluted in 60 μL
126 Buffer AVE (RNase free water containing 0.04% (w/v) sodium azide). No DNase or

127 RNase treatment was used. Following DNA extraction, samples were quantified on
128 the Qubit® 2.0 Fluorometer (Life Technologies, Paisley, UK) using the Qubit®
129 dsDNA HS assay kit (Life Technologies). Samples were stored at -20°C as 60 µL
130 aliquots for up to four weeks.

131 **DNA extraction from buffy coat**

132 For *RHD* intronic SNP sequencing, gDNA was extracted from buffy coats using the
133 QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions.
134 DNA was eluted in 200 µL Buffer AE and incubated at room temperature for 5 min
135 before centrifugation at 11 865xg for 1 min. For the *RHD* long-range PCR (LR-
136 PCR), gDNA was extracted from buffy coats using the Gentra® Puregene® Blood kit
137 (Qiagen) according to the manufacturer's instructions for RNA-free DNA. As the
138 buffy coat contained red blood cells, RBC Lysis Solution was used. Each sample
139 was eluted by adding 300 µL of DNA hydration solution and mixed vigorously for 5
140 seconds, followed by incubation at 65°C for 1 hour. The tube was then incubated at
141 room temperature overnight with gentle shaking in order to mix the gDNA with the
142 DNA hydration solution. Finally, the pure gDNA was transferred into a new 1.5 mL
143 tube and stored at -20°C. Following DNA extraction, samples were quantified on the
144 Qubit® 2.0 Fluorometer (Life Technologies) using the Qubit® double-stranded DNA
145 (dsDNA) High Sensitivity assay kit (Life Technologies).

146 **PCR Primers and Probes for dPCR**

147 Two multiplex reactions were tested on the QX100™ droplet digital PCR (ddPCR)
148 platform (Bio-Rad Laboratories, Herfordshire, UK) for *RH* zygosity testing (Table 1),
149 as previously described in Sillence *et al.* (13). The oligonucleotide sequences (High
150 Performance Liquid Chromatography (HPLC) purified, Eurofins Genomics, Germany)

151 and amplicon sizes for all target (FAM-labelled) and reference (HEX-labelled)
152 regions are shown in Table 1. Prior to zygosity testing, primer annealing
153 temperatures (56°C to 60°C) were optimised for both multiplex reactions (see
154 Supplemental Figure 1). The results in Supplemental Figure 1a showed successful
155 droplet separation of the *RHD5* (FAM) target at all annealing temperatures, but the
156 *AGO1* (HEX) reference showed sub-optimal separation at 60°C. Droplet separation
157 for the *RHD7* (FAM)/ *AGO1* (HEX) multiplex reaction (see Supplemental Figure 1b)
158 demonstrated the same pattern as previously discussed for the *RHD5* (FAM)/*AGO1*
159 (HEX) multiplex reaction. However, the optimal ratio was visible at 58.4°C (0.995).
160 Therefore 58°C was determined to be the optimum annealing temperature for both
161 multiplex reactions.

162 **dPCR**

163 The dPCR reactions were conducted in duplicate and run on the QX100™ Droplet
164 Generator (Bio-Rad) following manufacturer's instructions (see Sillence *et al.* (13)).
165 Plasma extracted samples were not diluted and a standard volume of template DNA
166 (5 µL) was added. Samples extracted from buffy coat were diluted and 50ng of DNA
167 was added to each 20 µL reaction and a non-template control (NTC) was included in
168 each assay.

169 **Data Analysis for dPCR**

170 The raw fluorescent data from the ddPCR platform was analysed using the Bio-Rad
171 QuantaSoft v1.2 software. Once thresholds for each sample had been set manually
172 using the 1D amplification plot, positive and negative droplets were determined (see
173 Supplemental Figure 1). The concentration was then determined by the software
174 using Poisson statistics (95% confidence interval) for each sample. The ratio of the

175 target (*RHD5-FAM* and *RHD7-FAM*) over the reference (*AGO1-HEX*) for each
176 sample was calculated as follows: FAM (copies/ μ L)/HEX (copies/ μ L). All statistical
177 analysis was performed using Mann Whitney U Test (SigmaPlot Version 12.5) and
178 significance was accepted at $p < 0.05$.

179 ***RHD* LR-PCR and Next generation sequencing (NGS)**

180 gDNA samples from blood donors of different phenotypes were tested using LR-PCR.
181 Three PCR products were designed to cover the entire *RHD* gene (Table 2). The
182 HPLC-purified primers were from Eurofins MWG Operon (London, United Kingdom).
183 The PCR reaction contained a final 1x concentration of PrimeSTAR GXL Buffer
184 (Takara, Japan), 200 μ M dNTP mixture, 0.2 μ M of each primer and 1.25 unit
185 PrimeSTAR GXL Polymerase per 50 μ L and 500ng DNA per reaction. A two-step
186 protocol was performed as 25 cycles of 98°C for 10 s and 68°C for 24 min, final hold
187 at 4°C. The amplicons were purified on 0.5% w/v agarose gel in 1X TAE buffer. The
188 long amplicons were purified by Agencourt® AMPure® XP beads (Beckman Coulter,
189 High Wycombe, UK) to ensure removal of primer dimers, polymerase and free
190 nucleotides. The samples were eluted in 50 μ L nuclease-free water. Purified
191 amplicons were quantified by Qubit® dsDNA Broad-Range assay kit (Life
192 Technologies) to allow the starting concentration of the sequencing libraries to be
193 100 ng. Following quantification, enzymatic fragmentation was completed using the
194 Ion Xpress™ Plus Fragment Library Kit (Life Technologies) resulting in fragments of
195 ~200bp. Next, the fragments were ligated with barcoded adapters, which add about
196 70bp to the fragments. P1 and Ion Xpress™ Barcode X adapters from the Ion
197 Xpress™ Barcode Adapters Kit (Life Technologies) were used to distinguish the
198 samples when pooled prior to sequencing. The adapter-ligated library was size
199 selected by SPRIselect® reagent kit (Beckman Coulter, High Wycombe, UK). After

200 each step (fragmentation, ligation and size selection), purification was conducted
201 using magnetic beads and the integrity, size distribution, concentration and quality of
202 the library in those steps was checked using the Agilent® 2100 Bioanalyzer®
203 instrument and Agilent High Sensitivity DNA Kit (Agilent Technologies UK Limited,
204 Stockport, UK).

205 Template-positive ion sphere particles (ISPs) containing clonally amplified DNA were
206 prepared by the Ion Personal Genome Machine™ (PGM™) Template OT2 200 Kit
207 (for 200 base-read libraries) (Life Technologies) with the Ion OneTouch™ 2 System.
208 Then the percentage of template-positive ISPs was checked by the Ion Sphere™
209 Quality Control assay (Life Technologies, Paisley, UK) on the Qubit® 2.0
210 Fluorometer (Life Technologies) and then enriched by the Ion OneTouch™ ES
211 Instrument before loading onto a 316™ chip. Sequencing was carried out using the
212 Ion PGM™ Sequencing 200 Kit v2 (Life Technologies) and the Ion Torrent PGM™.

213 **Bioinformatics for *RHD* LR-PCR**

214 Torrent Suite™ Software Version 4.4 was utilised in order to generate a summary
215 sequencing report indicating the number of reads generated by the sequencer, the
216 percentage of chip loading and the sequencing files. The FastQC software was run
217 to assess the quality control across the reads generated (17). The sequencing
218 samples were aligned to the human genome reference sequence (hg19) using the
219 Binary Alignment/Map (BAM) and were visualised using Integrative Genome Viewer
220 (IGV) Version 2.3.46.

221 The samples were annotated using the Variant Call Format (VCF) files to obtain the
222 SNPs and indels to analyse the genotype and predict the phenotype. Antigens were
223 determined by choosing the right transcript according to the Blood Group Antigen

224 Factsbook (18). Each antigen was determined by its chromosomal location, the type
225 of variant (SNP or indel), gene, the reference nucleotide, the changing nucleotide,
226 depth of coverage, the transcript used in analysis based on the NCBI database, the
227 location of the variant (intronic or exonic), codon, an exon number of that variant, an
228 amino acid substitution and the position of the nucleotide change. The SeattleSeq
229 Annotation tool 141 site was used to annotate the sequencing data of the LR-PCR
230 approach (19). By using Browser Extensible Data (BED) files, the bedtools website
231 was used to mask the *RHCE* gene in order to analyse the *RHD* gene (20). The
232 *RHCE* gene was annotated by 'Ns' on its sequencing nucleotides.

233 ***RHD* Intronic SNP sequencing**

234 gDNA samples from blood donors of different phenotypes were tested. *RHD*-specific
235 primers amplified the regions around the intronic SNPs (Table 2). Two different
236 enzymes were used, BioMix™ 2X master mix (Bioline Reagents Limited, United
237 Kingdom) or Q5® Hot Start High-Fidelity 2X Master Mix, (New England Biolabs,
238 United Kingdom). A 50 µL PCR reaction was prepared containing 1X master mix,
239 200ng of DNA template, 1 µM of each of the primers. Cycling was carried out on a
240 Veriti Thermal Cycler (Life Technologies) following optimised conditions; 95°C for 10
241 min, 35 cycles of 95°C for 30 s and optimised annealing temperature for 1 min, 72°C
242 for 30 s, followed by a final 72°C step for 10 min. To validate PCR amplification, PCR
243 products were run on a 1% w/v agarose gel in 1x TAE buffer. PCR products were
244 purified using the QIAquick Gel Extraction Kit, (Qiagen Ltd, West Sussex, United
245 Kingdom) according to the manufacturer's instructions. PCR amplicons were
246 subjected to Sanger sequencing by Eurofins Genomics. Results were aligned with
247 the human genome reference sequence (hg19). CodonCode Aligner 6.0 software
248 was used to analyse the data.

249 Results

250 Determination of *RHD* Zygosity

251 For zygosity testing, the presence or absence of *RHD* amplification on the ddPCR
252 platform was used to determine whether the samples were *RHD* negative or *RHD*
253 positive, respectively. The mean number of copies per droplet for all molecules was
254 0.15 (0.03 – 0.57) for plasma DNA samples and 0.39 (0.05 – 0.69) for buffy coat
255 DNA samples. The ratio of *RHD5* (FAM)/ *AGO1* (HEX) and *RHD7* (FAM)/ *AGO1*
256 (HEX) generated by the QuantaSoft v1.2 Software was then used to determine
257 whether the D-positive samples were hemizygous or homozygous for the *RHD* gene.
258 Samples showing ratios close to 1 were determined to be homozygous *RHD* positive
259 and samples with ratios closer to 0.5 were classified as hemizygous *RHD* positive
260 (Figure 2).

261 The results demonstrated that the assay worked equally well on cell free DNA and
262 gDNA for zygosity determination (Table 3) (Figure 2). Three rr control samples were
263 tested (147J, 1660, 7807) and results demonstrated amplification of only the
264 reference (*AGO1*), giving a ratio of zero (Figure 2). The hemizygous D+ R₀r
265 (Dce/dce) ($n = 8$), R₁r (DCe/dce) ($n = 12$) and R₂r (DcE/dce) ($n = 1$) samples
266 demonstrated ratios close to 0.5 as expected (Table 3) (Figure 2), except for sample
267 1777. Sample 1777, previously classified by serology as being phenotypically R₁r
268 (DCe/dce), expressed ratios of 0.97 and 1.04 for the *RHD5* and *RHD7* multiplex
269 reactions, respectively (Table 3). This result contradicted previous serological
270 classification and indicated that the sample expressed two copies of the *RHD* gene.
271 Therefore, it is more feasible that this sample actual expresses the R₁R₀ (DCe/Dce)
272 phenotype. The homozygous D+ R₁R₁ (DCe/DCe) ($n = 13$), R₂R₂ (DcE/DcE) ($n = 5$),

273 R₁R₂ (DcE/DcE) (*n* = 10) and R₂R₂ (DcE/DcE) (*n* = 1) samples were expected to
274 generate a ratio close to 1, and this was achieved in 90% of samples. Sample 087W
275 was serologically typed as expressing the R₂R₂ (DcE/DcE) phenotype. However, the
276 dPCR results demonstrate that this sample is hemizygous for the *RHD* gene, since
277 both assays illustrated a ratio close to 0.5 (Figure 2). Therefore, it is likely that
278 sample 087W has the R₂r'' (DcE/dcE) genotype as opposed to the R₂R₂ (DcE/DcE)
279 serologically predicted genotype. Further sequencing analysis was required to
280 determine the actual genotype of the incorrectly labelled R₁R₂ samples (729M and
281 351D) (Figure 2).

282 ***RHD* intronic polymorphisms**

283 We sequenced the complete *RHD* gene from individuals with defined *RH* genotypes
284 using LR-PCR (Table 2) and we identified several intronic polymorphisms that
285 closely correlated with the individuals *DCE* status. On further analysis using Sanger
286 sequencing, five SNPs showed complete concordance when scrutinised using
287 primers flanking these regions (Tables 2 and 4).

288 **Comparison of *RHD* intronic polymorphisms and zygosity**

289 Two of the R₁R₂ (DcE/DcE) presumed genotype samples tested (729M and 351D)
290 expressed ratios close to 0.5 for both assays (Figure 2b). Since sample 729M has
291 also been typed as weak D, it is highly unlikely that this sample is homozygous *RHD*
292 positive. Therefore, it is clear this sample has been misclassified as R₁R₂, but we
293 could not ascertain whether the true genotype for sample 729M was R₂r' (DcE/dCe),
294 R₂r (DCE/dce), R₀r^γ (Dce/dCE) or R₁r'' (DcE/dcE). Consequently, LR-PCR coupled
295 with NGS revealed that sample 729M displayed the exon 9 Gly385Ala 1154G>C
296 SNP, and thus was classified as weak D type 2. In addition, the sample illustrated

297 multiple *RHD* intronic SNPs which appear to be associated with the R₂ (DcE)
298 haplotype, which demonstrates that sample 729M is likely to be R₂r' (DcE/dCe)
299 (Table 4). Sample 351D was not typed serologically as weak D but the dPCR data
300 shows that only one copy of *RHD* is present (Figure 2b) and thus the genotype must
301 either be R₂r' (DcE/dCe), R_Zr (DCE/dce), R₀r^γ (Dce/dCE) or R₁r'' (DCe/dcE). This
302 sample did not show the R₂ associated *RHD* intronic SNPs and hence is likely to
303 have a genotype of R₁r'' (DCe/dcE), R₀r^γ (Dce/dCE) or R_Zr (DCE/dce).

304 **Discussion**

305 *RHD* zygosity assignment has proved to be a useful diagnostic tool in the clinical
306 management of HDFN. Here, determination of homozygous (*RHD/RHD*) fathers
307 would give confidence (assuming paternity) of prenatal prediction of D-positive
308 fetuses, and signal where further monitoring or administration of prophylactic anti-D
309 maybe required. Without doubt, the most appropriate technique would be the
310 assessment of D-positive infants directly by analysis of free fetal DNA in maternal
311 plasma. However, in repeat pregnancies fathered by *RHD/RHD* homozygotes
312 maternal plasma testing would not be necessary as the fetus would invariably be D-
313 positive. This is of course with the caveat that paternity can be assured during the
314 maternal consenting process. Previous methods have utilised qPCR (4, 7-9), MLPA
315 (6), mass spectrometry (10) and analysis of the *Rhesus box* (2, 5, 11). However, as
316 we have previously mentioned, individuals have been described that confound
317 zygosity testing when relying on analysis of the *Rhesus box* repeat sequences (11).
318 Here we describe a rapid and accurate further method for defining *RHD* zygosity.
319 We have used this on a small cohort of phenotyped blood samples and
320 demonstrated that this method could be used effectively to define paternal zygosity,

321 and in addition, to correct presumed phenotype in blood donors which is presently
322 dependent on phenotype prediction.

323 In three samples we have analysed, and a weak D sample, we have clearly
324 demonstrated homo and hemizyosity for *RHD*, which was not in concordance with
325 predicted phenotype. The vast majority of current genotyping methods (22-27) are
326 not able to define zygoty (except the study by Gassner *et al.* (10) or unless an
327 assessment of intronic *RHD*-specific SNPs is performed, some of which are
328 described in this paper). Our description of candidate SNPs that define the *RHD*
329 gene within the DcE haplotype will also provide a method to differentiate homo or
330 hemizyosity, and we have candidate *RHD* intronic SNPs that define the DcE and
331 Dce *RHD* genes (in preparation). However, much more work on a larger number of
332 donors (including the testing of rare RH haplotypes) has to be done before these
333 candidate *RHD* intronic SNPs can be confirmed as being truly DcE and Dce specific.
334 Nevertheless, these *RHD* intronic SNPs may not be able to differentiate between
335 DcE/DcE and DcE/dce; DcE/DcE and DcE/dce; and Dce/Dce and Dce/dce
336 genotypes, however, the dPCR method described here is able to facilitate this
337 (differentiating homo and hemizyosity). Clearly, for these candidate SNPs to have
338 clinical utility, a larger cohort of phenotyped samples will require sequencing. We
339 have subsequently performed such an analysis on 37 Rh phenotyped individuals,
340 and have found complete concordance with the five DcE-associated candidate SNPs
341 described in this study. We have identified a further 11 such candidate SNPs that
342 also are in concordance with DcE genotype. (WAT, KAS, AJH, MK, TEM and NDA,
343 manuscript in preparation). We are currently investigating a number of Rh variants
344 and rare phenotypes (e.g. Rz) to assist in their identification.

345 This method provides a quick and accurate platform for rapid determination of *RHD*
346 zygosity. In this small cohort of samples, we would be unlikely to see rare haplotypes
347 such as DCE, dCe, dcE and Dce. However, both dCe and dcE haplotypes were
348 identified. Further zygosity-based studies are clearly necessary to reassess the
349 population frequencies of these D-negative haplotypes. It is important also to
350 consider that fathers that are *RHD* hemizygous DCE/dCe or DcE/dcE may pass the
351 dCe or dcE haplotypes to their children, and these fetuses may be at risk of HDFN
352 due to anti- C or G (28) or anti-E (29). Fetal genotyping for inheritance of both Rh C
353 and Rh E has been routinely performed using maternal plasma and should therefore
354 be used in such cases where hemizyosity has been defined. We believe that the
355 method we describe here is a useful addition to the diagnostic repertoire available to
356 the clinician in the management of HDFN.

357

358 **References**

- 359 1. Avent ND, Reid ME. The Rh blood group system: a review. *Blood* 2000;95(2):375-
360 87.
- 361 2. Wagner FF, Flegel WA RHD gene deletion occurred in the Rhesus box. *Blood*
362 2000;95: 3662-8.
- 363 3. The Rhesusbase. The RHD Mutation Database [Online]. Available at:
364 <http://www.rhesusbase.info/> (Accessed October 2016).
- 365 4. Chiu RW, Murphy MF, Fidler C, Zee BC, Wainscoat JS, Lo YD. Determination of
366 RhD zygosity: comparison of a double amplification refractory mutation system
367 approach and a multiplex real-time quantitative PCR approach. *Clin Chem.*
368 2001;47(4):667-72.
- 369 5. Grootkerk - Tax MG, Maaskant - van Wijk PA, Van Drunen J, Der Schoot V, Ellen
370 C. The highly variable RH locus in nonwhite persons hampers RHD zygosity
371 determination but yields more insight into RH - related evolutionary events.
372 *Transfusion* 2005;45(3):327-37.
- 373 6. Haer - Wigman L, Veldhuisen B, Jonkers R, Lodén M, Madgett TE, Avent ND, et
374 al. RHD and RHCE variant and zygosity genotyping via multiplex ligation -
375 dependent probe amplification. *Transfusion* 2013;53(7):1559-74.
- 376 7. Perco P, Shao CP, Mayr WR, Panzer S, Legler TJ. Testing for the D zygosity with
377 three different methods revealed altered Rhesus boxes and a new weak D type.
378 *Transfusion* 2003;43(3):335-9.

- 379 8. Pirelli KJ, Pietz BC, Johnson ST, Pinder HL, Bellissimo DB. Molecular
380 determination of RHD zygosity: predicting risk of hemolytic disease of the fetus and
381 newborn related to anti - D. *Prenatal Diag* 2010;30(12 - 13):1207-12.
- 382 9. Krog GR, Clausen FB, Dziegiel MH. Quantitation of RHD by real-time polymerase
383 chain reaction for determination of RHD zygosity and RHD mosaicism/chimerism: an
384 evaluation of four quantitative methods. *Transfusion* 2007;47: 715-22.
- 385 10. Gassner C, Meyer S, Frey BM, Vollmert C Matrix-Assisted Laser
386 Desorption/Ionisation, Time-of-Flight Mass Spectrometry–Based Blood Group
387 Genotyping—The Alternative Approach. *Transfusion Medicine Reviews* 2013;27(1):
388 2–9.
- 389 11. Matheson KA, Denomme GA. Novel 3' Rhesus box sequences confound RHD
390 zygosity assignment. *Transfusion* 2002;42(5):645-50.
- 391 12. Daniels G. Rh and RHAG Blood Group Systems. In: Daniels G, editor. *Human*
392 *Blood Groups*. 3rd Ed. Oxford (UK): Wiley-Blackwell; 2013 p.185. 13. Sillence KA,
393 Roberts LA, Hollands HJ, Ross Welch C, Thompson HP, Kiernan M, et al. Fetal sex
394 and *RHD* genotyping with digital PCR demonstrates greater sensitivity than real-time
395 PCR. *Clin Chem* 2015;239:137.
- 396 14. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Analysis of the size
397 distributions of fetal and maternal cell-free DNA by paired-end sequencing. *Clin*
398 *Chem* 2010;56(8):1279-86.
- 399 15. Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high
400 throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD

401 immunoglobulin in RhD negative pregnant women: prospective feasibility study. Brit
402 Med J 2008;336(7648):816-8.

403 16. Legler TJ, Maas JH, Köhler M, Wagner T, Daniels GL, Perco P, Panzer S. RHD
404 sequencing: a new tool for decision making on transfusion therapy and provision of
405 Rh prophylaxis. Transfusion Med 2001;11:383-388.

406 17. Andrews, S. *FastQC: a quality control tool for high throughput sequence data*
407 (2010) [Online]. Available at:
408 <http://www.bioinformatics.babraham.ac.uk/projects/fastqc> [Accessed October 2016].

409 18. Reid ME, Lomas-Francis C, Olsson ML The Blood Group Antigen FactsBook, 3rd
410 Ed. Boston, Academic Press; 2012.

411 19. Seattleseq Annotation Tool 141. *SeattleSeq Variation Annotation* [Online].
412 Available at: <http://snp.gs.washington.edu/> (Accessed October 2016).

413 20. Bedtools. *bedtools: a powerful toolset for genome arithmetic* [Online]. Available
414 at: <http://bedtools.readthedocs.org/> (Accessed October 2016).

415 21. National Center for Biotechnology Information NCBI). dbSNP Short Genetic
416 Variations [Online]. Available at
417 https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs (Accessed October 2016).

418 22. Hashmi G, Shariff T, Zhang Y, Cristobal J, Chau C, Seul M, et al. Determination
419 of 24 minor red blood cell antigens for more than 2000 blood donors by high-
420 throughput DNA analysis. Transfusion 2007;47(4):736-47.

421 23. Avent ND, Martinez A, Flegel WA, Olsson ML, Scott ML, Nogues N, et al. The
422 Bloodgen project of the European Union, 2003–2009. *Transfus Med and Hemoth*
423 2009;36(3):162-7.

424 24. Avent ND. Large-scale blood group genotyping—clinical implications. *Brit J*
425 *Haematol* 2009;144(1):3-13.

426 25. Flegel WA, Gottschall JL, Denomme GA. Implementing mass-scale red cell
427 genotyping at a blood center. *Transfusion* 2015;55(11):2610-5.

428 26. Boccoz SA, Le Goff GC, Mandon CA, Corgier BP, Blum LJ, Marquette CA
429 Development and Validation of a Fully Automated Platform for Extended Blood
430 Group Genotyping. *The Journal of Molecular Diagnostics* 2016;18(1)

431 27. López M, Apraiz I, Rubia M, Piedrabuena M, Azkarate M, Veldhuisen B, Vesga
432 MA, van Der Schoot E, Puente F, Tejedor D Performance evaluation study of ID
433 CORE XT, a high throughput blood group genotyping platform. [Blood Transfus.](#) 2016:
434 Nov 25:1-7. doi: 10.2450/2016.0146-16. [Epub ahead of print]

435 28. Hadley A, Poole G, Poole J, Anderson N, Robson M. Haemolytic Disease of the
436 Newborn Due to Anti-G. *Vox Sang* 1996;71(2):108-12.

437 29. Rouillac C, Kim CLV, Blancher A, Roubinet F, Cartron JP, Colin Y. Lack of G
438 blood group antigen in DIIIb erythrocytes is associated with segmental DNA
439 exchange between RH genes. *Brit J Haematol* 1995;89(2):424-6.

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446 **Table 1:** *RHD5*, *RHD7* and *AGO1* oligonucleotide sequences, product size and gene
 447 location.

Amplicon location	Multiplex Reaction	Primer	Sequence (5' - 3')	Dual-Labelled Hydrolysis Probe (5' - 3')	Length (bp)
1p36.11 <i>RHD</i> Exon 5	1	<i>RHD5</i> Forward*	CGCCCTCTTCTTG TGGATG	FAM- TCTGGCCAAGTTTCA ACTCTGCTCTGCT-BHQ1	82bp
		<i>RHD5</i> Reverse*	GAACACGGCATTCTTCCTTTC		
1p36.11 <i>RHD</i> Exon 7	2	<i>RHD7</i> Forward*	CAGCTCCATCATG GGCTACAA	FAM- AGCTTGCTGGGTCTG CTTGGAGAGATC-BHQ1	75bp
		<i>RHD7</i> Reverse*	AGCACCAGCAGCA CAATGTAGA		
1p34.3	1 and 2	<i>AGO1</i> Forward**	GTTCCGGCTTTTAC CAGTCT	HEX- CTGCCATGTGGAAGATG ATG -BHQ1	81bp
		<i>AGO1</i> Reverse**	CTCCATAGCTCTC CCCACTC		

448 *Taken from Finning *et al.* (15)

449 **Taken from Fan, *et al.* (14)

450

451 **Table 2:** *RHD* intronic SNP and *RHD* long-range PCR oligonucleotide
 452 sequences, product sizes and corresponding SNP in the *RHD* gene (hg19
 453 human genome reference sequence, for intronic SNPs).

454

Intronic SNPs	Intron	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'	Length (bp)
25,611,580 G>A	2	TTTTACTGGACAGCCCTACT CC	CATGGCTATTTATTGTCTA GCAGCA	558
25,614,400 C>G	2	GCTACCATGCCCTGCTAAT	TCCAGTACTTTTCAGAGC C	417
25,625,471 T>C	3	GGGGCAGCTTCATCTTATC AAGAG	CTCACTGCAACCTCCACC CGTT	419
25,627,066 C>G	3	TGGGATTACAGGCAAATT AG	AGGTGTGACTTGAAGCCA T	834
25,648,349 T>C	8	TCCAGGAATGACAGGGCT	TGAGGACTGCAGATAGGG	525
<i>RHD</i> exons covered				
1-3	1,2	GATTGGGTCCGTGATTGGC ATT	GGCCGCGGGAATTCGATT GTTGTCTTTATTTTCAA ACCCT	22,829
2-7	2-6	GCCGCGAATTCAGTAGTGT GACGAGTGAACTCTATCT CGAT (Ds2-s*)	GGCCGCGGGAATTCGATT GAGGCTGAGAAAGGTTAA GCCA	23,610
7-10	7-9	GCCGCGAATTCAGTAGTGA CAAACCTCCCGATGATGTG AGTG	GGCCGCGGGAATTCGATT GTGGTACATGGCTGTATT TTATTG	22,731

455

456 *Adapted from Legler *et al.* (16)

457

458 **Table 3:** Zygosity testing results determined by ratio analysis for DNA samples
 459 extracted from both the plasma (cfDNA) and buffy coat (gDNA) of human whole
 460 blood samples.

Sample	RH ¹	Ratio (RHD5 (FAM) / AGO1 (HEX))	Ratio (RHD7 (FAM) / AGO1 (HEX))	Hemizygous or homozygous	Genotype determined by dPCR**	
147J*	rr (dce/ dce)	0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)	
1660*		0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)	
7807*		0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)	
9763*	R ₀ r (Dce/ dce)	0.45	0.43	Hemizygous	R ₀ r (Dce/ dce)	
069F*		0.5	0.49	Hemizygous	R ₀ r (Dce/ dce)	
740B*		0.47	0.46	Hemizygous	R ₀ r (Dce/ dce)	
258D*		0.51	0.51	Hemizygous	R ₀ r (Dce/ dce)	
(079*)*		0.51	0.50	Hemizygous	R ₀ r (Dce/ dce)	
649B*		0.5	0.5	Hemizygous	R ₀ r (Dce/ dce)	
8931*		0.49	0.49	Hemizygous	R ₀ r (Dce/ dce)	
5784*		0.49	0.50	Hemizygous	R ₀ r (Dce/ dce)	
065S*		0.49	0.49	Hemizygous	R ₁ r (DCE/ dce)	
118Z*	R ₁ r (DCE/ dce)	0.5	0.49	Hemizygous	R ₁ r (DCE/ dce)	
1226*		0.52	0.51	Hemizygous	R ₁ r (DCE/ dce)	
1306*		0.51	0.53	Hemizygous	R ₁ r (DCE/ dce)	
1777*		0.97	1.04	Homozygous <i>RHD</i> positive	R ₁ R ₀ (DCE/ Dce)	
180H*		0.52	0.52	Hemizygous	R ₁ r (DCE/ dce)	
181F*		0.52	0.49	Hemizygous	R ₁ r (DCE/ dce)	
148R ²		0.50	0.50	Hemizygous	R ₁ r (DCE/ dce)	
6418 ²		0.51	0.49	Hemizygous	R ₁ r (DCE/ dce)	
3093		0.51	0.51	Hemizygous	R ₁ r (DCE/ dce)	
572R ²		0.50	0.50	Hemizygous	R ₁ r (DCE/ dce)	
7687 ²		0.50	0.51	Hemizygous	R ₁ r (DCE/ dce)	
5481 ²		R ₂ r (DcE/ dce)	0.50	0.51	Hemizygous	R ₂ r (DcE/ dce)
1220*		R ₁ R ₁ (DCE/ DCE)	0.98	1.01	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)
131Z*			0.99	1.04	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)
165F*	0.94		0.9	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
1793*	0.99		1	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
0670*	0.91		0.85	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
1347*	0.99		1.03	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
138R*	0.95		0.98	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
052M	0.99		1.03	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
247X	1.02		1.01	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
078U	0.99		1.01	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
103N	1.01		1.03	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
1461	0.99		1.01	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
877L	1.01		0.98	Homozygous <i>RHD</i> positive	R ₁ R ₁ (DCE/ DCE)	
658G	R ₂ R ₂ (DcE/ DcE)		1.02	1.03	Homozygous <i>RHD</i> positive	R ₂ R ₂ (DcE/ DcE)
738W			1.02	1.04	Homozygous <i>RHD</i> positive	R ₂ R ₂ (DcE/ DcE)
087W			0.51	0.49	Hemizygous	R ₂ r** (DcE/ dcE)

132H		1.01	1.03	Homozygous <i>RHD</i> positive	R ₂ R ₂ (DcE/ DcE)	
689U		0.99	1.01	Homozygous <i>RHD</i> positive	R ₂ R ₂ (DcE/ DcE)	
729M ²		0.50	0.49	Hemizygous	R ₁ r ⁺ (DcE/ dcE) or R ₂ r ⁺ (DcE/ dcE)	
896H		0.98	1.03	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
898D		0.99	0.97	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
351D		0.51	0.51	Hemizygous	R ₁ r ⁺ (DcE/ dcE) or R ₂ r ⁺ (DcE/ dcE)	
9316	R ₁ R ₂ (DcE/ DcE)	1.02	1.01	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
911E		1.02	1.03	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
4195		1.02	1.01	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
645C		1.06	1.03	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
3627		0.99	1.01	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
8873		1.02	1.03	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)	
746P		R ₂ R _z (DcE/ DCE)	1.02	0.99	Homozygous <i>RHD</i> positive	R ₁ R ₂ (DcE/ DcE)

461 ¹ Serologically predicted phenotype provided by National Health Service Blood and Transplant (NHS
462 BT) (Bristol, UK).

463 ² Sample is Weak D.

464 *DNA samples tested from plasma.

465 ** The C/c and E/e status based on serological information. Only the D/d genotype was corrected by
466 dPCR.

467

468 **Table 4:** *RHD* intronic SNP sequencing and *RHD* LR-PCR NGS results for a range
 469 of DNA samples.

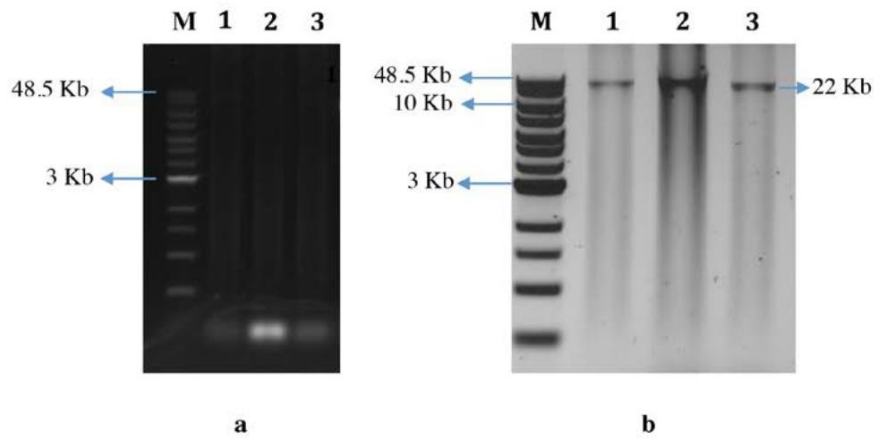
Intronic SNPs in <i>RHD</i> (R ₁ /R ₁ to R ₂)	<i>RHD</i> Intron	R ₁ R ₁ (DcE/ DcE) (n=4)	R ₁ r (DcE/ dce) (n=1)	R ₀ r (Dce/ dce) (n=8)	R ₂ R ₂ (DcE/ DcE) (n=6)	R ₂ r (DcE/ dce) (n=1)	R ₁ R ₂ (DcE/ DcE) (n=5)	Sample 729M (n=1)	Sample 351D (n=1)
25,611,580 G>A	2	G/G	G	G	A/A	A	G/A	A	G
25,614,400 C>G rs28718098*	2	C/C	C	C	G/G	G	C/G	G	C
25,625,471 T>C rs2904843*	3	T/T	T	T	C/C	C	T/C	C	T
25,627,066 C>G rs2986167*	3	C/C	C	C	G/G	G	C/G	G	C
25,648,349 T>C rs28669938*	8	T/T	T	T	C/C	C	T/C	C	T

470 * Taken from the National Center for Biotechnology Information (NCBI) (16).

471 The table indicates the serologically inferred genotype of the samples provided by
 472 the National Health Service Blood and Transplant (NHS BT) (Bristol, UK).

473

474 **Figure 1:** LR-PCR products for the Rh blood group system. Three long-range
475 amplicons (1, 2, and 3) were designed to amplify the entire *RHD* gene. (a) An RhD-
476 negative sample shows no bands for the *RHD* LR-PCR in lanes 1, 2 and 3, which
477 represent the three amplicons. (b) An RhD-positive sample gives amplification of all
478 three products, with each product being about 22 kb.

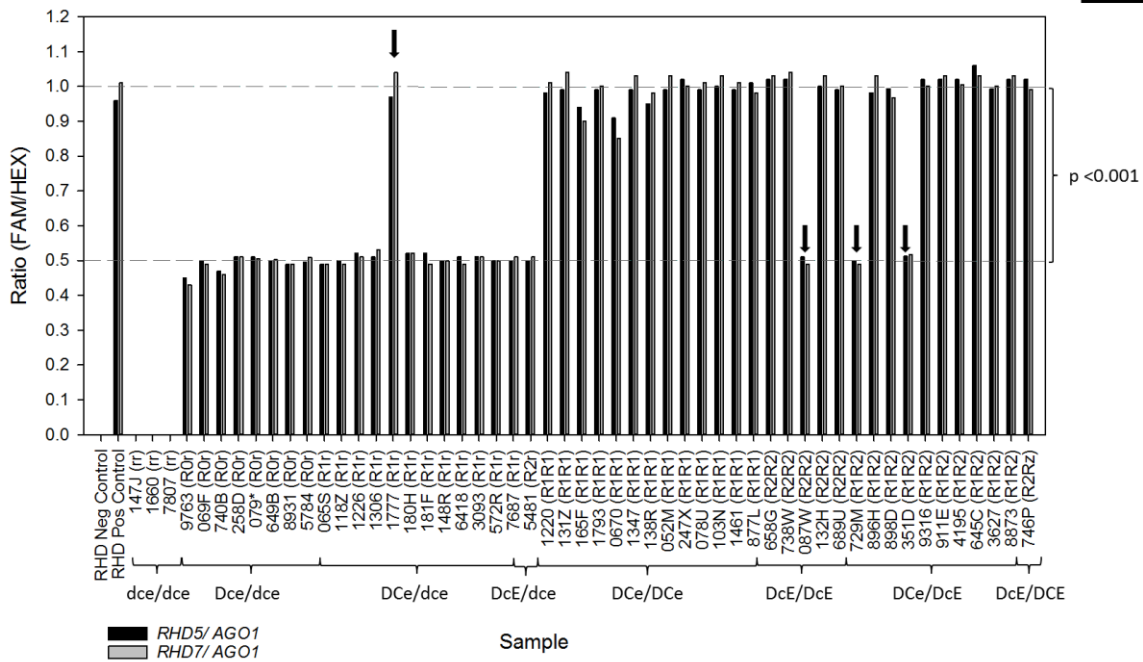


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482 **Figure 2:** Ratio analysis to determine zygosity using two multiplex reactions (*RHD5*
 483 (*FAM*)/*AGO1* (*HEX*) and *RHD7* (*FAM*)/*AGO1* (*HEX*) for samples with varying Rh
 484 phenotypes. The grey dotted lines at 0.5 and 1 on the y axis represent the ratio
 485 generated by hemizygous D+ samples and homozygous D+ samples, respectively.
 486 The mean ratio for hemizygous and homozygous D+ positive samples for both
 487 plasma and buffy coat extracted samples (Table 3) illustrated significant difference
 488 ($p < 0.001$). The arrows indicate the samples that illustrated discordant results
 489 compared with the serologically predicted genotype.



490

491

492 **Supplemental Figure 1:** Annealing temperature gradient (60°C, 58.4°C, 57.4°C and
493 56°C) of dPCR for both multiplex reactions (*RHD5* (FAM)/*AGO1* (HEX) and *RHD7*
494 (FAM)/*AGO1* (HEX)) using sample 0745 (homozygous for *RHD*) extracted from
495 human whole blood and a NTC. a) Optimisation of the *RHD5* (FAM) and *AGO1*
496 (HEX) multiplex reaction. The results illustrate that separation is visible for both
497 targets at all annealing temperatures. Marginally greater separation is visible at
498 56°C, but all annealing temperatures are no more than 0.03-0.04 away from a ratio
499 of 1. b) Optimisation of the *RHD7* (FAM) and *AGO1* (HEX) multiplex reaction. The
500 results illustrate that separation is visible for both targets at all annealing
501 temperatures. However, optimal separation was determined to be 58.4°C, since this
502 temperature expressed a ratio closer to 1 (0.995).

