

1 **Minor groove binder modification of widely used TaqMan hydrolysis probe for detection**  
2 **of dengue virus reduces risk of false-negative real-time PCR results for serotype 4**

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21

22 **Summary**

23 Dengue is a vector-transmitted viral infection that is a significant cause of morbidity and  
24 mortality in humans worldwide, with over 50 million apparent cases and a fatality rate of 2.5% of  
25 0.5 million severe cases per annum in recent years. Four serotypes are currently co-circulating.  
26 Diagnosis of infection may be by polymerase chain reaction, serology or rapid antigen test for

27 NS1. Both pan-serotype and serotype-specific genome detection assays have been described,  
28 however, achieving adequate sensitivity with pan-serotype assays has been challenging.  
29 Indeed, as we show here, inspection of components and cycling parameters of a pan-serotype  
30 RT-qPCR assay in use in laboratories worldwide revealed insufficient probe stability to  
31 accommodate potential nucleotide mismatches, resulting in false-negatives. A minor-groove  
32 binder (MGB)-modified version of the probe was designed and its performance compared with  
33 that of the original probe in 32 samples. Eight of the samples were undetected by the original  
34 probe but detected by the MGB modified probe and six out of seven of these that could be  
35 serotyped belonged to serotype 4. Sequencing of the region targeted by the probe in these  
36 samples revealed two mismatches which were also universally present in all other serotype 4  
37 sequences in a public database. We therefore recommend adoption of this MGB modification in  
38 order to reduce the risk of false-negative results, especially with dengue serotype 4 infections.

39

40 **Keywords:** dengue, TaqMan, false-negative, sensitivity, serotype 4, minor groove binder

41

42 **Abbreviations:**

43 MGB = minor groove binder

44 qPCR = quantitative polymerase chain reaction

45 RT-qPCR = reverse transcription quantitative polymerase chain reaction

46  $C_q$  = quantification cycle (sometimes referred to as  $C_t$  or threshold cycle)

47

48 **1. Introduction**

49

50 Dengue, caused by a member of the family *Flaviviridae*, is the most common arboviral infection  
51 worldwide. Globally, case incidence is thought to be 50-100 million per annum, though the true  
52 number may be higher as not all infections are apparent and misclassification may occur in

53 regions with a high burden of febrile illness (Bhatt et al., 2013; Stanaway et al., 2016). Clinical  
54 infections range widely in severity from asymptomatic to dengue haemorrhagic fever and death.  
55 Other than supportive therapy no specific treatment is available and as yet there is no effective  
56 vaccine universally recommended for those at risk (Aguilar et al., 2016; Halstead, 2017; World  
57 Health Organization, 2017). A vaccine would need to prevent infection in the infection-naïve,  
58 such as travellers to dengue-endemic regions, and neonates in endemic countries, as well as  
59 those who have experienced primary infection. Recommendations for the only licensed vaccine  
60 currently available (the Pasteur CYD-TDV) are limited to those aged 9-45, who are likely to have  
61 previously been exposed to infection (Sridhar et al., 2018; Wilder-Smith et al., 2018).

62

63 Four serotypes co-circulate in most endemic countries, and these are genetically variable  
64 (Costa et al., 2012). Initial infection with any serotype gives type-specific lifelong immunity,  
65 however, prior infection with one serotype can predispose to a more serious episode on  
66 subsequent infection with a different serotype (Halstead et al., 1970; Katzelnick et al., 2017).

67

68 Diagnosis of dengue is on clinical suspicion based on symptoms and travel history; in addition a  
69 wide variety of laboratory methods are in current use. Blood samples may be analysed for  
70 RNA, IgM and IgG or NS1 antigen; the presence of RNA and/or IgM suggesting acute infection.  
71 Point of care diagnostic tests are also available, although the sensitivity of these tests when  
72 performed in the field has been variable (Pang et al., 2017; Sa-ngamuang et al., 2018).

73 Molecular techniques are widely used, though the resource requirements for these tests mean  
74 that their implementation may not be feasible in resource-limited settings. Molecular tests to  
75 detect viraemic infections include qualitative/nested PCR, reverse-transcription quantitative  
76 PCR (RT-qPCR) and isothermal amplification methods (Domingo et al., 2010; Poersch et al.,  
77 2005). Some tests seek to differentiate serotypes for epidemiological purposes, while others  
78 are intended to be pan-serotype. Serotyping of dengue infection has limited applicability in

79 immediate clinical management. However, epidemiological surveys are important to assess  
80 whether a predominant serotype in a locale is supplanted, bringing with it the risk of secondary  
81 infections and more severe consequences.

82  
83 A widely cited pan-serotype RT-qPCR assay published in 2002 (Drosten et al., 2002) that is  
84 apparently used by laboratories in many countries (Domingo et al., 2010) was identified in a  
85 literature search and evaluated using archived samples. An initial failure to detect one of four  
86 samples tested led to a closer inspection of this assay and, as a result, to its improvement  
87 through minor groove binder (MGB) modification of its TaqMan probe (i.e. 5' hydrolysis probe).  
88 The MGB-modified and un-modified probe (herein designated the 'original' probe) assays were  
89 tested on a larger group of samples and two sequence mismatches between the 'original' probe  
90 and dengue serotype 4 sequences were identified. The MGB modification was thus shown to  
91 prevent false-negative results being generated with serotype 4 samples.

92

93

## 94 **2. Materials and Methods**

95

### 96 *2.1 Samples*

97 Samples #1-21: Archived EDTA-plasma samples from University College London Hospital that  
98 had been submitted for arbovirus testing between January and August 2016 were reviewed. 21  
99 samples that had been reported elsewhere as positive for dengue virus RNA were identified and  
100 retrieved from storage at -20 °C The total storage time at -20 °C varied between 5 and 14  
101 months prior to nucleic acid extraction (mean 10 months). Samples #22-32: Archived samples  
102 were stored at -80 °C since 2012 (Samples #22, 26-30), 2016 (Samples #23-25) or 2018  
103 (Samples #31-32). All samples from Brazil were processed at the Flavivirus Laboratory,  
104 Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

105

## 106 *2.2 Ethical Approval*

107 The study was approved by the local University College London Hospital Clinical Governance  
108 committee following regulatory requirements for diagnostic assay development for Samples #1-  
109 21 and the Ethical Screening Committee of Fiocruz (CAAE): 90249218.6.1001.5248 (2.998.362)  
110 for Samples #22-32.

111

## 112 *2.3 Nucleic acid extraction*

113 RNA was extracted from plasma samples manually using the Qiagen viral RNA mini kit (Qiagen,  
114 Hilden, Germany) according to the manufacturer's instructions (except volumes as follows) or  
115 the Qiagen EZ1 BioRobot. Sample input and elution volumes for manual extraction were  
116 matched to the BioRobot for the samples from UCLH: 200 µl sample input and 90 µl elution.  
117 For samples from Brazil, only 100µl was available, which was supplemented with 100µl  
118 negative normal human serum, and extracted as described. Extracted RNA was stored at -  
119 80°C.

120

## 121 *2.4 Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)*

122 Real-time RT-qPCR was performed in accordance with the MIQE guidelines (Bustin et al.,  
123 2009) using TaqMan Fast Virus 1-Step Master Mix (cat. no. 4444432, Life Technologies,  
124 Paisley, UK), primers at 600 nM and probe at 100 nM (sequences as in (Drosten et al., 2002)  
125 and Figure 1, synthesized by Sigma, Haverhill, UK); either a 6-carboxyfluorescein (FAM)/  
126 tetramethylrhodamine (TAMRA) probe for the 'original' assay (Drosten et al., 2002) or a FAM/  
127 minor groove binder (MGB)-non-fluorescent quencher probe (synthesized by Life Technologies)  
128 for the MGB-modified assay (Figure 1). Each well contained 15 µl total reagents with 1 µl RNA  
129 extract, and each condition was run in triplicate wells. Cycling conditions (ABI 7500 thermal  
130 cycler, Applied Biosystems) were: 50 °C 5 mins, 95 °C 20 secs, then 45 cycles of 95 °C 3 secs,

131 60 °C 1 min. Negative controls (H<sub>2</sub>O) were run on every plate. Data were analysed using the  
132 ABI 7500 software v2.0.6.

133

#### 134 *2.5 Synthesis of RNA by in vitro transcription*

135 Transcripts used to generate the dengue RNA dilution series were synthesised using the T7  
136 MegaScript kit (Life Technologies) and PCR amplicons as template, amplified from Sample #14  
137 (serotype 2). RNA from this clinical samples was initially reverse transcribed using Superscript  
138 IV and random hexamers (both Life Technologies). Amplified DNA template was produced by  
139 nested PCR using primers EG273/EG250 then EG295/EG296 (Table 1) and Platinum SuperFi  
140 polymerase (cat. no. 12351010, Life Technologies). Transcripts were cleaned up using the  
141 MegaClear kit (Life Technologies) and the yield assessed using the Nanodrop One<sup>c</sup> (Thermo  
142 Scientific, Paisley, UK).

143

#### 144 *2.6 Sequencing*

145 RNA extracts were reverse transcribed using Superscript IV with reverse primer EG244 (Table  
146 1) (Samples #2, #3, #6-17 and #19-21) according to the manufacturer's instructions. Where  
147 amplification by nested PCR was initially unsuccessful, random hexamers were used for the  
148 reverse transcription step (Samples #4 and #18). Nested PCR reactions were carried out on all  
149 samples using the primers in Table 1 and Platinum SuperFi Taq polymerase according to the  
150 manufacturer's instructions in 25 µl (first round) and 50 µl (second round) reactions. After the  
151 second round, 5 µl PCR reaction product was analysed by agarose gel electrophoresis to  
152 ensure unique bands of the correct size had been obtained, and DNA from the remaining 45 µl  
153 reaction product was purified by the QIAquick PCR purification kit (Qiagen). Bi-directional  
154 Sanger sequencing of the purified amplicon was carried out by Genewiz Ltd. (Essex, UK).

155

#### 156 *2.7 Serotyping*

157 To assign serotype to Samples #1-21, the segment that was sequenced (corresponding to  
158 10467-10660, numbering according to GenBank AF038403) was used as input for a nucleotide  
159 BLAST search ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and the serotype of the closest matched dengue  
160 sequences from the database noted. None of the sequences from this study matched more  
161 than one serotype. Samples #22-32 were assigned a serotype using the method described in  
162 (Santiago et al., 2013).

163

### 164 *2.8 Sequence analyses to explore probe/target mismatches in serotype 4 samples*

165 Analysis of .ab1 files, alignment to template and inspection of sequences was carried out using  
166 DNADynamo (Blue Tractor Software Ltd).

167

168 To create a multiple sequence alignment, all whole virus genome sequences from samples  
169 taken from a human host, classified as dengue serotype 4 that included data between  
170 nucleotides 10635 and 10701 (the qPCR amplicon from (Drosten et al., 2002), numbering  
171 according to AF038403) were downloaded from the Virus Pathogen Database and Analysis  
172 Resource ([www.viprbrc.org](http://www.viprbrc.org)), aligned using MUSCLE via the online interface, then opened in  
173 Seqotron (Fourment and Holmes, 2016) for further analysis. This dataset comprised 192  
174 genomes. The section encompassing the 'original' assay primers and probe was selected and  
175 extraneous sequence data removed, as well as any genomes with missing data for this section.  
176 The resulting FASTA file contained 147 sequences from geographically diverse locations. To  
177 create Supplementary Table 1, the percent identity of each nucleotide at each position was  
178 calculated using consensus mode in DNADynamo.

179

## 180 **3. Results**

181

### 182 *3.1 A widely used pan-serotype RT-qPCR assay can yield false-negative results*



205 therefore investigated further. The melting temperatures ( $T_m$ ) of the oligonucleotides were  
206 calculated by several alternative methods as shown in Table 2. These calculations revealed  
207 that the  $T_m$  of the probe was approximately the same as that of the primers. This was a  
208 surprising finding given that TaqMan probe design guidelines state that the probe  $T_m$  should be  
209 about 10 °C higher than that of the primers (Bustin, 2000). If the probe  $T_m$  is too low then stable  
210 binding may not occur before displacement and hydrolytic cleavage by the DNA polymerase  
211 during amplification. An inadequate  $T_m$  difference between that of the probe and that of the  
212 primers would be likely to make probe hybridization unstable in the face of even minor  
213 sequence mismatch.

214

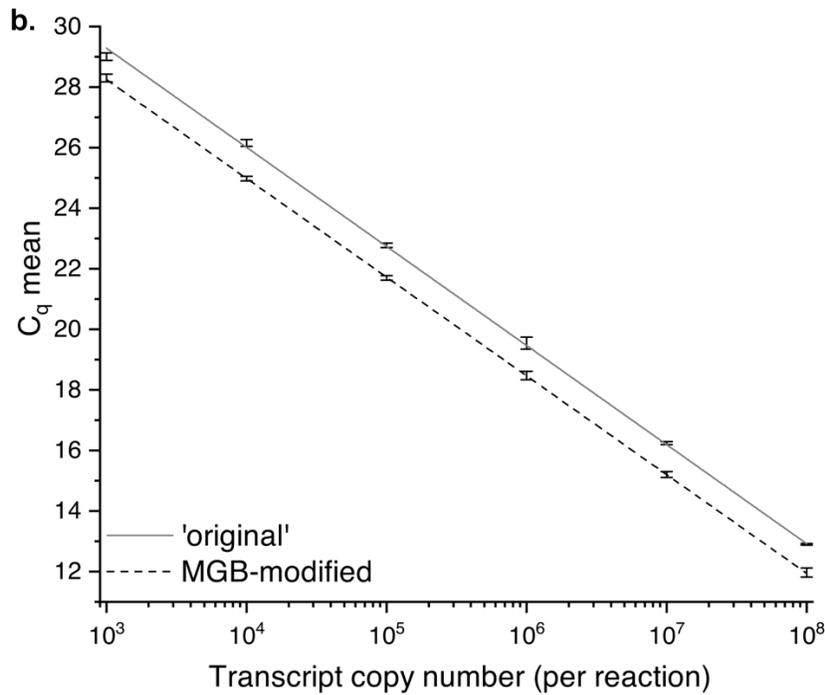
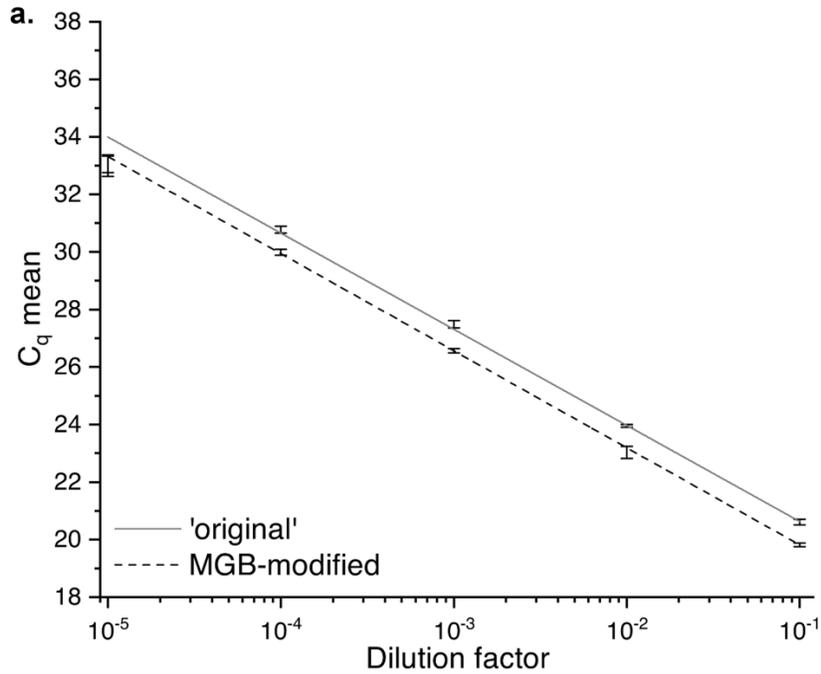
### 215 *3.2 MGB-modified probe has optimised characteristics and reduces risk of false-negatives*

216 Heterogeneity in the bases upstream of the probe sequence, and the proximity of the reverse  
217 primer downstream of the probe, meant that extending the probe to increase its  $T_m$  was not an  
218 option. To optimise the assay we therefore elected to synthesise an alternative probe of  
219 identical sequence and length but with a 3' minor groove binder (MGB) moiety. This stabilises  
220 the duplex, increasing the  $T_m$  of the probe by between 10 and 20 °C, without lengthening it  
221 (Garson et al., 2012). This alternative probe was synthesised (EG242) and the assay was  
222 designated 'MGB-modified'.

223

224 To investigate whether adding the MGB modification to the probe made a difference to assay  
225 characteristics (PCR efficiency and detection limit) a dilution series of a sample with a high viral  
226 load (Sample #14) was prepared. Ten-fold serial dilutions were made and analysed by both the  
227 MGB-modified and the 'original' assays in triplicate (Figure 2a). The fitted regression line for the  
228 MGB-modified probe showed detection at approximately 1  $C_q$  lower than the 'original' probe  
229 across most of the dilution range (Figure 2a). The slopes, -3.33 and -3.17, representing a

230 nominal PCR efficiency of ~99.7 % and ~106.6 %, and the R squared values (0.998 and 0.994)  
231 for the MGB-modified and 'original' assays respectively, were not significantly different. The  
232 dilution series was repeated a second time with RNA transcript generated from Sample #14 to  
233 assess the minimum copy number detectable by the two assays (Figure 2b). Again, fitted  
234 regression lines for the MGB-modified probe showed detection at approximately 1 C<sub>q</sub> lower than  
235 the 'original' probe across most of the dilution range, equivalent to approximately a 2-fold  
236 increase in sensitivity (Figure 2b). The limit of qualitative detection for both assays was  
237 approximately 100 RNA copies per reaction, though the limit of quantitation was ~1000 copies.  
238 Similar slopes, nominal PCR efficiencies and R squared values were seen (-3.26 and -3.24,  
239 ~102.5 % and ~103.4 %, 1.000 and 0.999 for the MGB-modified and 'original' assays  
240 respectively) (Figure 2b).  
241



242

243 **Figure 2. Amplification of samples generated from ten-fold serial dilutions.**

244 Each dilution was analysed in triplicate using both the MGB-modified probe and the 'original'

245 probe. Error bars represent the standard deviation of the mean of positive wells. a) Ten-fold

246 serial dilutions were made with Sample #14 nucleic acid extract, covering the dilution range

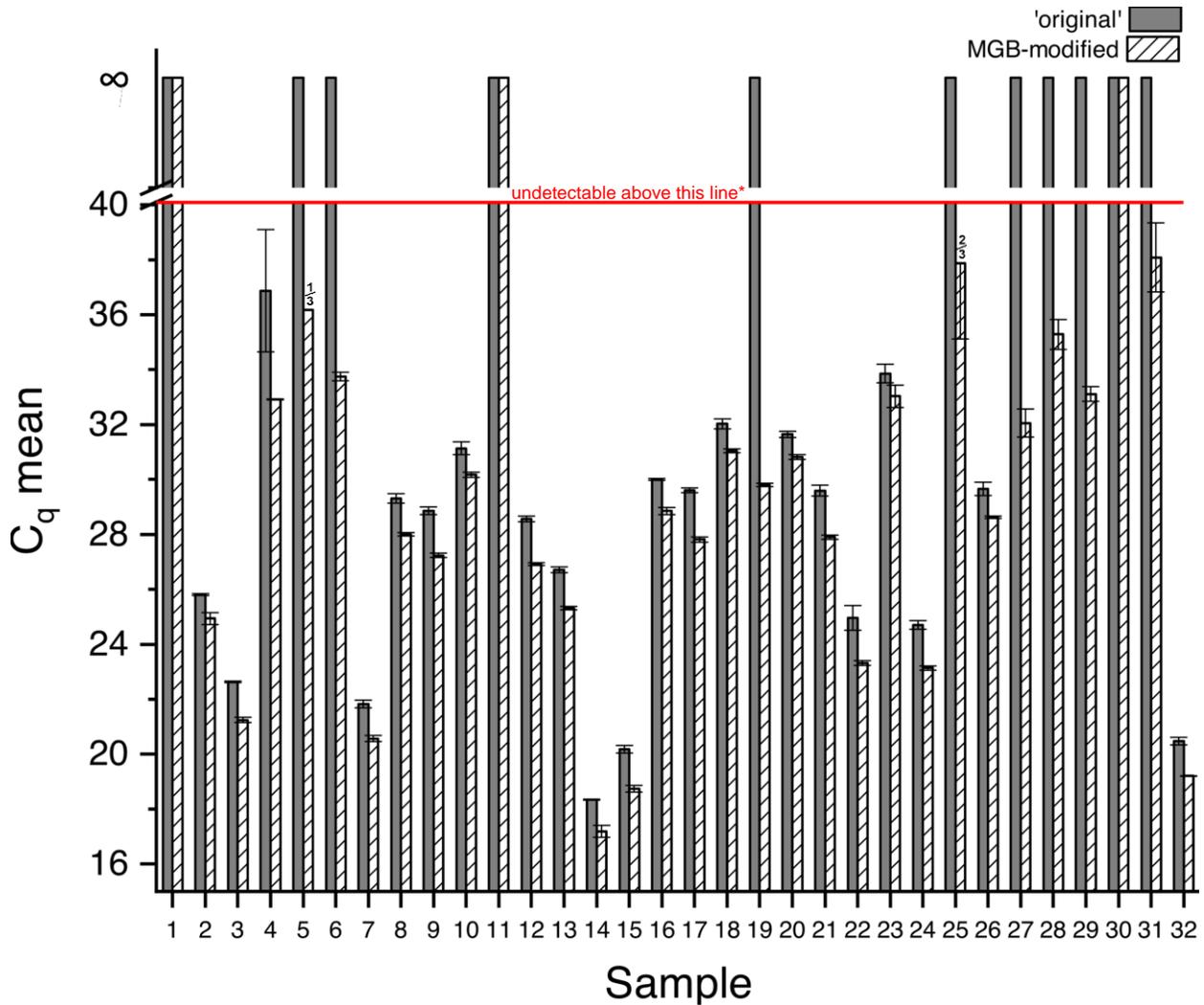
247 from  $10^{-1}$  to  $10^{-8}$ . The nominal PCR efficiency,  $R^2$ , and slope for the MGB-modified assay were  
248 99.7 %, 0.998 and -3.33, and for the 'original' assay were 106.6 %, 0.994 and -3.17. b) Ten-fold  
249 serial dilutions were made with RNA transcript generated from Sample #14 template. Between  
250  $10^0$  and  $10^8$  copies per sample were tested; for both assays, only samples with  $10^2$  copies or  
251 more yielded a positive result. The nominal PCR efficiency,  $R^2$ , and slope for the MGB-modified  
252 assay were 102.5 %, 1.000 and -3.26, and for the 'original' assay were 103.4 %, 0.999 and -  
253 3.24.

254

255 To compare the performance of the MGB-modified and 'original' assays on a wide variety of  
256 samples, 32 archived samples reported elsewhere as dengue RNA-positive were tested.

257 Overall, 29 of 32 samples were found to be positive with the MGB-modified assay but only 21 of  
258 32 with the 'original' assay. Sample #5 yielded one positive well out of three repeats and  
259 Sample #25 yielded two positive wells out of three repeats with the MGB-modification but zero  
260 of three were positive with the 'original' probe for both these samples. For every positive  
261 sample, the  $C_q$  with the MGB-modified assay was approximately one cycle lower than generated  
262 by the 'original' assay (Figure 3 and Table 3).

263



264

265 **Figure 3. A comparison of  $C_q$  values obtained with 32 archived samples assayed by the**  
 266 **MGB-modified and the 'original' assay.**

267 Each sample was analysed in triplicate; error bars represent the standard deviation of the mean  
 268 of positive samples. If fewer than 3 of 3 wells yielded positive results (Samples #5 and #25) ,  
 269 the number of positive wells is given above each bar. Samples for which 3/3 wells were  
 270 negative are shown with  $C_q$  mean above 40 cycles.

271

272 *3.3 False-negative results with 'original' probe were from serotype 4 samples*

273 To determine whether dengue serotype may be associated with the inability of the 'original'  
 274 assay to detect certain samples, a region between nucleotides 10467-10660 (numbering based



298 used to serotype all samples (Table 3). Samples #6 and #19 are serotype 4; Sample #14 is the  
299 high titre sample used to generate Figure 2 and is serotype 2. Mismatches to the assay primers  
300 and probe are shown with arrows and highlighted in bold. Outside of the primer/probe binding  
301 regions (delineated by horizontal lines), sequence differences between the samples are also  
302 highlighted in bold. There are two different reverse primers (R1 and R2 mixed 1:1) therefore  
303 although there are five variant sites between the two serotypes in this region, all have ~100%  
304 homology with one of the reverse primers.

305

#### 306 *3.4 Two mismatches in probe binding site are present in all available serotype 4 sequences*

307 We investigated whether the 'original' probe mismatches with serotype 4 samples found in this  
308 study are also found in previously sequenced samples from around the globe. A multiple  
309 sequence alignment was prepared from all available dengue serotype 4 genomes from the Virus  
310 Pathogen Database and Analysis Resource ([www.viprbrc.org](http://www.viprbrc.org)) with sequence data covering the  
311 entire forward primer and reverse primer binding region (n = 147 sequences). This  
312 demonstrated that the two mismatches found in the probe-binding region of Samples #6 and  
313 #19 in this study are in fact universally present in all available dengue serotype 4 genomes  
314 (Supplementary Table 1).

315

#### 316 *3.5 No false positive signals are seen in dengue-negative samples*

317 To ensure that the MGB-modified probe maintained its specificity and did not yield false positive  
318 results with other members of the family *Flaviviridae*, 16 samples from patients with hepatitis C  
319 and 22 samples from patients with Zika virus were tested *in vitro* by qPCR. None of the  
320 samples tested by qPCR yielded false-positive results. An *in silico* analysis using NCBI Primer-  
321 BLAST also failed to find any cross-reactivity with the human genome, or with the genomes of  
322 other members of the *Flaviviridae* including the following viruses: hepatitis C, chikungunya, Zika  
323 and yellow fever.

324

325 **4. Discussion**

326

327 The incidence of dengue is slowly rising worldwide and vigilance is increasingly required in non-  
328 endemic countries where dengue is imported by returning travellers (Neumayr et al., 2017;  
329 Stanaway et al., 2016). As yet, there is no vaccine that can be universally recommended and  
330 no cure, and morbidity and mortality remains high in countries that can least afford the dual  
331 burdens of healthcare costs and loss of human capital. A clear and accurate understanding of  
332 incidence is key to obtaining a full epidemiological picture of the disease, and accurate  
333 diagnostic tools are essential. In this study, we have shown how the sensitivity of a pan-  
334 serotype RT-qPCR assay, employed to diagnose viraemic dengue infection can be significantly  
335 improved by MGB modification of the TaqMan hydrolysis probe. Dengue serotype 4 comprises  
336 a significant minority of infections worldwide, and therefore the diagnostic tools chosen by  
337 laboratories must reliably detect this serotype. In the quality assessment study of 2010  
338 (Domingo et al., 2010), samples of dengue serotype 4 were included at a level of  $10^5$  genome  
339 equivalents per mL, two orders of magnitude higher than the minimum detection requirement to  
340 achieve an 'acceptable' classification in this exercise. Despite this relatively high level, 14/46  
341 laboratories failed to classify the serotype 4 sample as dengue positive. Several pan-serotype  
342 dengue assays proposed in the literature include more than one primer (or probe) to ensure  
343 detection of dengue serotype 4 (Alm et al., 2014; Dumoulin et al., 2008; Santiago et al., 2013).  
344 Interestingly, the modification to the (Drosten et al., 2002) assay proposed by Dumoulin et al. is  
345 the addition of a second probe identically located but with two nucleotides changes to map  
346 exactly the dengue serotype 4 consensus sequence (Dumoulin et al., 2008). When the  
347 (Drosten et al., 2002) assay was originally published in 2002, MGB-probes were only just  
348 becoming available; their widespread use not common until a few years later. It is therefore

349 appropriate to update this assay using technological advances that have become available  
350 since that time.

351

352 The MGB modification acts as a molecular clamp, increasing the affinity of the probe for its  
353 complementary sequence and hence increasing the  $T_m$ . In this way, the qualities of the probe  
354 can be modified without changing sequence or length. This has been previously shown to make  
355 assays more tolerant of mismatches between the probe and target sequence and can raise the  
356  $T_m$  of the probe to around 10 °C higher than the  $T_m$  of the primers (Garson et al., 2012; Nolan et  
357 al., 2006). While the effect on detection of the serotype 4 samples was dramatic in terms of  
358 preventing false-negatives, the detection of other serotypes was not impaired, and indeed,  
359 appeared to be improved by a mean of about one cycle compared to the non-MGB probe.  
360 There is therefore a clear case for laboratories that use the 'original' assay to switch to using an  
361 MGB-modified probe, which will increase reliability of detection of serotype 4 samples without  
362 adversely affecting detection of serotypes 1, 2 and 3 or compromising specificity.

363

364 In terms of the travel history of patients from whom Samples #1-21 derived, more than half had  
365 been to Thailand or Indonesia (including Bali), in line with previous data from travellers from  
366 Europe with dengue (Neumayr et al., 2017) (Supplementary Table 2). Samples #22-32 were  
367 from patients resident in Brazil. Out of the 32 total samples, seven were found to be serotype 4  
368 and of the 21 European samples, two were found to be serotype 4. This is also broadly in line  
369 with sentinel European laboratory data that found this serotype to be the least commonly  
370 isolated (Neumayr et al., 2017). Both of the sequenced viruses isolated from these samples  
371 had two mismatches with the probe (out of 20 nucleotides), in common with all serotype 4  
372 sequences available in a public database that covered this section of the genome. Whilst these  
373 mismatches might not prevent very highly viraemic samples from being detected, they would be

374 likely to cause a false-negative result to be returned if the level of viraemia was moderate or  
375 low.

376

377 Although all archived samples from UCLH analysed here had been reported as dengue RNA-  
378 positive when tested as fresh samples, they had been stored at -20 °C for up to 14 months  
379 (average of 10 months) and due to clinical requirements, had been subjected to freeze thaws  
380 before commencing the present study. The Brazilian samples were stored at -80 °C after  
381 collection. We speculate that the storage at -20 °C , and/or a reduced input level of sample  
382 through both reduced extraction volume and reduced sample input to the RT-qPCR assay, may  
383 account for the failure of both the modified and the 'original' assays to detect Samples #1, #11,  
384 and #30 and for the difficulty in obtaining amplicons for sequencing from Samples #1 and #5.

385

## 386 **5. Conclusion**

387

388 In conclusion, we have shown that an MGB-modification of a TaqMan probe from a widely used  
389 RT-qPCR assay can improve the sensitivity of the assay for dengue serotype 4 samples, which  
390 contain two mismatches with respect to the probe sequence. In light of this, we would suggest  
391 that laboratories that use the 'original' assay to detect dengue viraemia use the MGB-modified  
392 probe to avoid false-negative results from infections with dengue serotype 4.

393

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398

399 **Author contributions:**

400 EG, EN and JG designed the study. EG, JH, RF and PS performed experiments. EN provided  
401 samples. EG and JG analysed data and wrote the paper. All authors had the opportunity to  
402 review the manuscript before submission.

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413

414 **Competing interests:**

415 Declarations of interest: none.

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