Development and evaluation of a loop-mediated isothermal amplification (LAMP) assay for the detection of *Tomato brown rugose fruit virus* (ToBRFV)

Alian Sarkes[1], Heting Fu[1], David Feindel[1], Michael W. Harding[2], and Jie Feng[1]

[1] Alberta Plant Health Lab, Crop Diversification Centre North, Alberta Agriculture and Forestry, Edmonton, AB, T5Y 6H3, Canada;

[2] Crop Diversification Centre South, Alberta Agriculture and Forestry, Brooks, AB, T1R 1E6, Canada

Correspondence to:

Michael Harding, email: michael.harding@gov.ab.ca

Jie Feng, email: jie.feng@gov.ab.ca

Abstract

Tomato brown rugose fruit virus (ToBRFV) is a member of *Tobamovirus* infecting tomato and pepper. Within North America, both the United States and Mexico consider ToBRFV to be a regulated pest. In Canada, the presence of ToBRFV has been reported, but an efficient diagnostic system has not yet been established. Here, we describe the development and assessment of a loop-mediated isothermal amplification (LAMP)-based assay to detect ToBRFV. The LAMP test was efficient and robust, and results could be obtained within 35 min with an available RNA sample. Amplification was possible when either water bath or oven were used to maintain the temperature at isothermal conditions (65°C), and results could be read by visual observation of colour change. Detection limit of the LAMP was eight target RNA molecules. Under the experimental conditions tested, LAMP was as sensitive as qPCR and 100 times more sensitive than the currently used rt-PCR. We recommend this sensitive, efficient LAMP protocol to be used for routine lab testing of ToBRFV.

30 **Introduction**

31 Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops in the world [1].
32 Canada is the main producer of greenhouse tomatoes in North America, with an annual yield of
33 more than 250,000 t and a value of around $500 million (Agriculture & Agri-Food Canada,
34 www.agr.gc.ca/pmc-cropprofiles). Diseases caused by viruses are one of the most critical factors
35 affecting tomato production worldwide [2]. *Tomato brown rugose fruit virus* (ToBRFV) is a
36 member of the *Tobamovirus* genus with a host range including tomato, pepper and some weeds
37 such as nightshades [3]. It was first detected in Israel in 2014 [4] and later in countries in North
38 America [5,6], Asia [7-9] and Europe [10,11]. Within North America, both the United States [12]
39 and Mexico [13] consider ToBRFV to be a regulated pest. In Canada, ToBRFV was first
40 identified in Ontario in 2019 (http://thegrower.org/news/tomato-brown-rugose-fruit-virus-
41 identified-ontario). Since then the Alberta Plant Health Lab (APHL) began testing of tomato
42 samples submitted from Alberta greenhouses, but no positive samples had been found at the time
43 this report was submitted.

44

45 ToBRFV has a single-stranded positive-sense RNA of ~6,400 nucleotides (nt), with a typical
46 *tobamovirus* genome organization that consists in four open reading frames (ORFs) encoding
47 two replication-related proteins [7]. On tomato, ToBRFV causes symptoms including leaf
48 interveinal yellowing and deformation, mosaic staining, young leaves deformation and necrosis,
49 sepal necrosis and deformation, young fruits discoloration, deformation and necrosis [3]. Besides
50 the significant damage to yield and fruit quality, ToBRFV is of special concern compared to
51 other *tobamoviruses* because of its ability to overcome the *R* genes *Tm-2* and *L*, which are
52 deployed in tomato and pepper varieties, respectively, for *tobamoviruses* resistance [4].

53

54 An effective disease management program is dependent on timely and proper identification to
55 the causal agent of the disease. Enzyme-linked immunosorbent assay (ELISA), reverse
56 transcriptase polymerase chain reaction (rt-PCR), next-generation sequencing (NGS) and
57 transmission electron microscopy (TEM) have been used for ToBRFV detection [3]. In Canada,
58 the Alberta Plant Health Lab, Alberta Agriculture and Forestry, had utilized the rt-PCR methods
59 developed by Ling et al. [6] for diagnosis on suspected tomato samples. Recently, protocols
60 employing real time rt-PCR for ToBRFV detection were developed and demonstrated to be
61 highly specific and sensitive [14].

62

63 As an alternative of the above mentioned diagnostic methods, loop-mediated isothermal
64 amplification (LAMP) was not yet available for ToBRFV. This technique was originally
65 designed by Notomi et al. [15], in which six specific primers named internal primers (F2-
66 F1c/B2-B1c), external primers (F3/B3) and loop-specific primers (LoopF/LoopB) were used.
67 Compared to other methods, LAMP has advantages due to its rapidity, specificity and simplicity.
68 For example, a typical LAMP reaction can be completed within 30 min, and it is highly specific
69 as it can detect six gene regions of the target sequence by the six primers. Furthermore, LAMP
70 does not need specialized equipment, such as a thermocycler, but can be conducted in warm
71 water bath, thermal blocks, or oven, and does not require electrophoresis for detection and
72 identification of the reaction product(s).

73

74 LAMP diagnostic protocols have been developed for many plant diseases [16-20]. However,
75 there was no such protocol available for ToBRFV. The purpose of the present study was to

76 develop a LAMP test for detection of ToBRFV and compare it with PCR and qPCR tests. Our
77 results indicated that the LAMP method was sensitive and specific with potential to be developed
78 into a field-friendly diagnostic test.
79
80 **Materials and Methods**
81 **Chemicals and standard techniques**
82 All chemicals and equipment were purchased from Fisher Scientific Canada (Ottawa, ON) unless
83 otherwise specified. Extraction of genomic DNA and total RNA from plant samples was
84 conducted using a DNeasy plant mini kit (Qiagen Canada, Toronto, ON) and a RNeasy plant
85 mini kit (Qiagen Canada), respectively. Synthesis of the first-strand cDNA from the extracted
86 total RNA was conducted using a QuantaBio qScript cDNA synthesis kit (VWR Canada,
87 Edmonton, AB). Primers and gBlocks were synthesized by Integrated DNA Technologies
88 (Coralville, IA). LAMP, polymerase chain reaction (PCR) and reverse transcriptase (rt)-PCR
89 were conducted in a Proflex 96-well PCR cycler. Quantitative (q) PCR was performed in a
90 CFX96 touch real-time PCR detection system (Bio-Rad Canada, Mississauga, ON).
91
92 **LAMP primer design and pre-selection**
93 A query of the national center for biotechnology information (NCBI) whole genome sequence
94 (WGS) database (https://www.ncbi.nlm.nih.gov/genome) with the term "tomato brown rugose
95 fruit virus" resulted in one entry (tested on Feb 25, 2020) with an International Nucleotide
96 Sequence Database Collaboration (INSDC) number KT383474. Based on this WGS, ten sets of
97 LAMP primers were designed using LAMP Designer
98 (http://www.premierbiosoft.com/isothermal/lamp.html), an online tool designing LAMP primers,

99 and screening them for specificity. Using the WGS of KT383474 as query, a BLASTn was
100 conducted against the NCBI database. From the result of the BLAST, all available ToBRFV
101 WGS (seven in total as confirmed by Feb 25, 2020), three selected TMV WGS and two selected
102 ToMV WGS were retrieved. Alignment of these 12 sequences was conducted using Clustal
103 Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). The output of the alignment was transferred
104 into a Microsoft Word file and the file was named Align.doc. Based on Align.doc, the specificity
105 of all primers in the ten primer sets was manually checked. If the last five nucleotides at the 3'
106 end of a primer matched with any of the non-targets (the three TMV and the two ToMV) or did
107 not match with one of the targets (the seven ToBRFV), the entire primer set that this primer
108 belongs to was discarded. After this pre-selection, three LAMP primer sets remained and one set
109 was selected for use in the subsequent studies. The selected primer set was hereafter referred as
110 LAMP primers and their locations and sequences are given in Fig. 1 and Table 1, respectively.
111

112 **Design of gBlocks and qPCR primers**

113 In the file Align.doc, one genomic region of 999 nt containing all binding sites of the LAMP
114 primers was selected. Three 999-nt double-stranded DNA fragments, corresponding to the RNA
115 sequence of this genomic region in one selected ToBRFV strain (INSDC no: KT383474; nt
116 1657-2655), one selected TMV strain (INSDC no: FR878069; nt 1649-2647) and one selected
117 ToMV strain (INSDC no: MH507166; nt 1653-2651) were synthesized as gBlocks. These
118 gBlocks were named $gBlock^{ToBRFV}$, $gBlock^{TMV}$ and $gBlock^{ToMV}$, respectively. In addition, two
119 gBlocks, in which the sequences of the binding site of primer F1c or B2 on $gBlock^{ToBRFV}$ were
120 replaced with the corresponding sequences in one of the ToMV strains (DQ873692), were
121 synthesized and named $gBlock^{\Delta F1c}$ and $gBlock^{\Delta B2}$, respectively (Table 1 and Fig. 1). Based on

122 the sequence of gBlock^{ToBRFV}, a pair of qPCR primers was designed using the Primer3 software
123 (http://primer3.ut.ee). This primer pair was name qF1/qR1 and their sequences are given in Table
124 1.
125
126 **LAMP assays**
127 All LAMP reactions were conducted in the WarmStart colorimetric LAMP master mix (NEB
128 Canada, Whitby, ON). Each reaction was 25 µL in total and contained 1 µL template. Quantities
129 of other components in each reaction followed the NEB's instructions for the master mix. All
130 reactions were conducted in individual 200-µL PCR tubes. The reaction program consisted of
131 only one step in which the tubes were incubated at 65°C for 30 minutes. After the incubation, the
132 reactions were checked visually and the results were recorded by photography.
133
134 **PCR, rt-PCR and qPCR**
135 Each reaction of PCR, rt-PCR and qPCR was 25 µL containing 1 µL template and 0.25 µM of
136 each primer. PCR and qPCR was conducted in Promega PCR master mix and SsoAdvanced
137 universal SYBR green supermix (Bio-Rad Canada), respectively. The PCR program consisted of
138 an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s,
139 annealing at 55°C for 45 s and extension at 72°C for 1 min, and a final extension at 72°C for 5
140 min. The qPCR program consisted of an initial denaturation step of 95°C for 2 min, followed by
141 40 cycles of 5 s at 95°C and 1 min at 60°C. After completion of the qPCR amplification, a
142 melting curve analysis was run to evaluate the amplification specificity. rt-PCR was conducted
143 with a OneStep RT-PCR kit (Qiagen Canada) by following the manufacturer's instructions.
144

145 **Assays on gBlocks**

146 Each gBlock was dissolved in water and the concentration was adjusted to 100 fM, which
147 roughly equals to 60000 molecules/µL. From this concentration a set of 10× serial dilutions were
148 prepared for each gBlock down to a concentration of 0.06 molecules/µL (seven solutions for
149 each gBlock). Using the serial dilutions of all gBlocks as templates, LAMP was conducted.
150 Using the serial dilutions of gBlock-ToBRFV as templates, PCR and qPCR were conducted with
151 primers ToBRFV-F/ToBRFV-R [6](Ling et al. 2019) and qF1/qR1 (Table 1), respectively. In all
152 assays, sterile, DNA-free water was used as the negative control. All LAMP, PCR and qPCR
153 were repeated using the same preparations of serial dilutions.

154

155 **Assays on genomic DNA from health plants**

156 Genomic DNA was extracted from young leaves of Tomato (var. Cherry Nebula), pepper (var.
157 Tabasco), potato (*Solanum tuberosum* var. Norland) and tobacco (*Nicotiana tabacum* unknown
158 variety). Using 40 ng of genomic DNAs as templates, LAMP was conducted individually for
159 each of the four host plants. The LAMP assay was repeated on alternative DNA preparations.

160

161 **Assays on RNA of infected plants**

162 Total RNA was extracted from tomato plants with symptoms of ToBRFV infection. Five RNA
163 samples, confirmed to be ToBRFV positive, were provided to APHL by the Plant Disease Clinic
164 of University of Guelph and named 'original RNA samples'. The APHL tested the five original
165 RNA samples again with rt-PCR using the primer pair ToBRFV-F/ToBRFV-R, and with LAMP
166 using the designed LAMP primers. In addition, a 2-µL subsample was taken from each of the
167 five original RNA samples; the five subsamples were pooled and diluted 30× to form a 300-µL

168 RNA mixture. From this mixture, a set of eight 10-fold dilution series was prepared. rt-PCR and
169 LAMP were conducted on all eight dilutions using the above indicated primers. In all assays,
170 sterile, RNA-free water was used as the negative control. All assays were repeated using the
171 same RNA preparations.
172
173 **Assays on cDNA derived from the infected plants**
174 A 2-µL subsample was taken from each of the five original RNA samples; the five subsamples
175 were pooled, from which the first-strand cDNA was synthesized and the final cDNA solution
176 was adjusted to 300 µL. From this cDNA solution, a set of eight 10-fold dilution series was
177 prepared. These eight dilutions were used as template for PCR, LAMP and qPCR reactions. The
178 primer pair ToBRFV-F/ToBRFV-R was used in PCR. Primer pairs ToB5520F/ToB5598R and
179 qF1/qF2 were used in qPCR. In all assays, sterile DNA-free water was used as the negative
180 control. All assays were repeated using the same cDNA preparations.
181
182 **Statistics**
183 In all qPCR assays, each sample was tested with three technical repeats. The average and
184 standard deviation of the quantification cycle (Cq) values from the three repeats were calculated
185 using MS Excel. Calculation of Poisson distribution was conducted with an online Poisson
186 distribution calculator (https://stattrek.com/online-calcµLator/poisson.aspx).
187
188 **Results**
189 **LAMP on the gBlock**

190 In all LAMP reactions, changing color of the reaction mixture from pink to yellow indicated a
191 positive result, which was hereafter referred to as positive signal. With the designed LAMP
192 primers, no positive signal could be generated from gBlock^{TMV} and gBlock^{ToMV} (Fig. 2A).
193 Positive signals were generated from gBlock^{ToBRFV} when its molecule number was more than or
194 equal to 6 per 25-µL reaction (Fig. 2A). When gBlock$^{\Delta F1c}$ and gBlock$^{\Delta B2}$ were used as the
195 templates, positive signals could be generated when their molecule numbers were more than or
196 equal to 600 per 25-µL reaction; there was no positive signal when the molecule numbers were
197 less than or equal to 60 per 25-µL reaction (Fig. 2B). These results confirmed that the LAMP
198 primers were ToBRFV-specific. When the 6 molecules/µL gBlock^{ToBRFV} sample was repeatedly
199 tested by LAMP, 35 out of 40 reactions were found to be positive (Fig. 2C). This result indicated
200 that the LAMP assay could detect as little as 6 molecules in a 25-µL reaction more than 87% of
201 the time. In contrast, PCR could detect the target when the molecule number of gBlock^{ToBRFV}
202 was more than or equal to 600 per 25-µL reaction (Fig. 2D). In qPCR assay, Cq values were
203 generated from all of the three repeated reactions when the molecule number of gBlock ToBRFV
204 was more than or equal to 6 per 25 µL; when the molecule number was 0.6 per 25 µL, only one
205 out of the three reactions generated a Cq value (Fig. 2E). These results indicated that the
206 sensitivity of the LAMP assay on the gBlock was similar to that of qPCR but 100 times higher
207 than that of PCR. Repeated assays of LAMP, PCR and qPCR produced results similar to Figs.
208 2A and 2C, Fig. 2D and Fig. 2E, respectively.
209

210 **LAMP on plant genomic DNA**

211 When genomic DNA from healthy plants of four *Solanaceae* species was used as the template in
212 LAMP, no positive signal could be generated (data not shown). This result confirmed the
213 specificity of the LAMP primers.
214
215 **LAMP on RNA samples**
216 When RNA was used as the template, LAMP generated positive signals from all five original
217 RNA samples (Fig. 3A). This result was confirmed by rt-PCR (Fig. 3B). When the serial
218 dilutions were used as the templates, LAMP could generate positive signals from the dilutions 1-
219 7 (Fig. 3C); in contrast, rt-PCR could only produce a band from dilutions 1-5 (Fig. 3D). This
220 result indicated that LAMP was 100 times more sensitive than rt-PCR on RNA samples.
221 Repeated assays of LAMP and PCR produced results similar to Figs. 3A and 3C and Figs. 3B
222 and 3D, respectively.
223
224 **Efficiency of LAMP on cDNA**
225 When the serial dilutions of cDNA were used as the templates, LAMP generated positive signals
226 from samples 1-6 dilutions (Fig. 4A). In contrast, PCR generated positive bands from samples 1-
227 4 (Fig. 4B). qPCR assays using alternative primer sets showed similar sensitivity as LAMP (Fig.
228 4C). Repeated assays of LAMP, PCR and qPCR produced results similar to Fig. 4A, Fig. 4B and
229 Fig. 4C, respectively. These results indicated that the sensitivity of the LAMP assay on the
230 cDNA samples was similar to that of qPCR but 100 times higher than that of PCR.
231
232 **Discussion**
233 **Specificity of the LAMP**

234 LAMP is more specific than other PCR-based techniques because LAMP has six specific
235 primers. In this study, all LAMP primers were designed to be specific to all ToBRFV strains
236 with an available WGS, and non-specific to TMV and ToMV. TMV and ToMV are closely
237 related to ToBRFV [3], which is supported by the fact that WGS of TMV and ToMV were
238 always the top BLAST hits when the NCBI database was queried with the ToBRFV WGS. When
239 the LAMP primers were tested on the gBlock^{TMV} and gBlock^{ToMV}, as well as genomic DNA
240 from various *Solanaceae* plants, no signal could be generated, further confirming the specificity
241 of the LAMP primers to ToBRFV. Moreover, when the LAMP primers were tested on
242 gBlock$^{\Delta F1c}$ and gBlock$^{\Delta B2}$, which differed from gBlock^{ToBRFV} by only on a few nt on the binding
243 sites of primer F1c and B2, respectively, no positive signal could be generated at concentrations
244 of ≤ 60 molecules/25µL. This result supported the conclusion that the LAMP primers were
245 highly specific to ToBRFV.
246
247 **Sensitivity of the LAMP**
248 On the serial dilutions of the gBlocks or cDNA, LAMP was 100 times more sensitive than rt-
249 PCR and similar in sensitivity as qPCR. As well, on the serial dilutions of the RNA samples,
250 LAMP was 100 times more sensitive than rt-PCR. These results were comparable with those of
251 LAMP testing on other RNA viruses. For examples, LAMP was 100 times more sensitive than
252 rt-PCR on the detection of *Citrus leaf blotch virus* [21] and *Little cherry virus 1* [22]; on *Potato*
253 *virus X* [23] and *Sugarcane streak mosaic virus* [24], LAMP was 10 times more sensitive than rt-
254 PCR. Compared to rt-qPCR, LAMP was similarly sensitive for detection of *Onion yellow dwarf*
255 *virus* [25] and *Potato leafroll virus* [26].
256

257 Testing primers on gBlocks allows more accurate calculation of primer efficiency. In this study,
258 we obtained data that 35 out of 40 LAMP reactions generated positive signals when the template
259 gBlock concentration was 6 molecules/μL. At a very low concentration, the distribution of DNA
260 particles in aliquots follows a Poisson distribution [27]. Using the Poisson distribution calculator,
261 we could calculate that the possibility of obtaining ≥ 4 gBlock molecules in a 1-μL aliquot from
262 a 6 molecules/μL solution was 85%, which is comparable to the data from the LAMP test (35/40
263 = 88%). Since gBlock is double-stranded and the virus RNA is single-stranded, we concluded
264 that the LAMP could detect the virus RNA in a concentration of as low as 8 viruses per reaction.
265 This is comparable to qPCR results obtained in this study and to the theoretical limit of qPCR
266 detection (3 molecules) proposed by Forootan et al. [28. The estimated efficiency of the RNA
267 extraction kit is approximately 40 to 80% (the ratio between the molecule numbers of extracted
268 genomic RNA and the number of virus particles in the sample) when the sample was 100-mg
269 tomato leaf tissue and the final RNA volume was 50 μL (Feng, unpublished data). Since 1 μL
270 template was used in the LAMP test, we concluded that the LAMP test could detect ToBRFV
271 when a 100-mg plant sample contains as little as 1,000 virus particles (8÷40%×50). While this
272 level of detection is encouraging, it is important to note that the minimal number of virus
273 particles necessary to cause plant infection could be very low. For example, one Tobacco etch
274 virus (TEV) particle is sufficient to initiate a systemic infection [29]. We want to emphasize here
275 that more improvement is required on the commonly used diagnostic protocols, and that the
276 efficiency of a molecular-based diagnostic protocol is determined not only by the efficiency of
277 the detection technique only, but also by other factors such as the efficiency of DNA/RNA
278 extraction.
279

280 **Time-saving and simplicity of LAMP**

281 Compared to PCR, rt-PCR or rt-qPCR, LAMP is time saving. For example, in the present study,
282 testing one DNA, RNA or cDNA sample by LAMP could be completed within 35 min. Testing
283 eight samples could be completed in less than one hour. In contrast, testing one or eight samples
284 by PCR or rt-PCR needed more than three hours (for PCR and electrophoresis) and testing by rt-
285 qPCR would need more than 2 hours. All LAMP results reported in the present study were
286 generated in a PCR thermocycler. However, identical results were obtained when the LAMP
287 tests were conducted in an oven or a heat block. Thus, for labs where PCR or qPCR
288 thermocycler is not available, LAMP protocol developed in this study is highly recommended for
289 ToBRFV testing.

290

291 **Acknowledgements**

292 We thank Dr. Shannon Xuechan Shan from the Plant Disease Clinic of University of Guelph for
293 providing all RNA samples used in this study. This study was supported by a grant from the
294 Canadian Agricultural Partnership (no. 601322) to JF.

295

296 **References**

297 1. Foolad MR, Panthee DR. Marker-assisted selection in tomato breeding. Crit Rev Plant Sci.
298 2012;31: 93-123.
299 2. Hanssen IM, Lapidot M, Thomma BP. Emerging viral diseases of tomato crops. Mol Plant-
300 Microbe Interact. 2010;23: 539-548.
301 3. Oladokun J, Halabi M, Barua, P, Nath PJPP. Tomato brown rugose fruit disease: current
302 distribution, knowledge and future prospects. Plant Pathol. 2019;68: 1579-1586.

303 4. Luria N, Smith E, Reingold V, Bekelman I, Lapidot M, Levin I, et al. A new Israeli
304 *Tobamovirus* isolate infects tomato plants harboring *Tm-22* resistance genes. PLoS One.
305 2017;12: e0170429.
306 5. Cambrón-Crisantos JM, Rodríguez-Mendoza J, Valencia-Luna JB, Rangel SA, De Jesús
307 García-Ávila C, López-Buenfil JA. First report of *Tomato brown rugose fruit virus*
308 (ToBRFV) in Michoacan, Mexico. Mex J Phytopathol. 2018;37: 185-192.
309 6. Ling KS, Tian T, Gurung S, Salati R, Gilliard A. First report of *Tomato brown rugose fruit*
310 *virus* infecting greenhouse tomato in the United States. Plant Dis. 2019;103: 1439.
311 7. Salem N, Mansour A, Ciuffo M, Falk BW, Turina M. A new *tobamovirus* infecting tomato
312 crops in Jordan. Arch Virol. 2016;161: 503-506.
313 8. Alkowni R, Alabdallah O, Fadda Z. 2019. Molecular identification of *Tomato brown rugose*
314 *fruit virus* in tomato in Palestine. J Plant Pathol. 2019;101: 719-723.
315 9. Fidan H, Sarikaya P, Calis O. First report of *Tomato brown rugose fruit virus* on tomato in
316 Turkey. New Dis Rep. 2019;39: 18.
317 10. Menzel W, Knierim D, Winter S, Hamacher J, Heupel M. First report of *Tomato brown*
318 *rugose fruit virus* infecting tomato in Germany. New Dis Rep. 2019;39: 1.
319 11. Panno S, Caruso AG, Davino S. First report of *Tomato brown rugose fruit virus* on tomato
320 crops in Italy. Plant Dis. 2019;103: 1443.
321 12. USDA. Federal order for U.S. imports of tomato (*Solanum lycopersicum*) and pepper
322 (*Capsicum* spp.) hosts of *Tomato brown rugose fruit virus* (ToBRFV). 2019.
323 https://www.aphis.usda.gov/import_export/plants/plant_imports/federal_order/downloads/20
324 19/DA-2019-28.pdf.

325 13. Puente M. AMSAC - Mexican Seed Trade Association. Presentation delivered at NAPPO
326 AGM. Interactions between government and industry facing the impacts of an emerging
327 pest-the case of ToBRFV. Wednesday, Oct. 30, 2019.
328 14. Panno S, Ruiz-Ruiz S, Caruso AG, Alfaro-Fernandez A, San Ambrosio MI, Davino S. Real-
329 time reverse transcription polymerase chain reaction development for rapid detection of
330 *Tomato brown rugose fruit virus* and comparison with other techniques. PeerJ. 2019;7:
331 e7928.
332 15. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-
333 mediated isothermal amplification of DNA. Nuc Acids Res. 2000;28: 63-70.
334 16. Kogovšek P, Mehle N, Pugelj A, Jakomin T, Schroers HJ, Ravnikar M. et al. Rapid loop-
335 mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-
336 vein homogenate has the same performance as qPCR. Eur J Plant Pathol. 2017;148: 75-84.
337 17. Okiro LA, Tancos MA, Nyanjom SG, Smart CD, Parker ML. Comparative evaluation of
338 LAMP, qPCR, conventional PCR, and ELISA to detect *Ralstonia solanacearum* in Kenyan
339 potato fields. Plant Dis. 2019;103: 959-965.
340 18. Romero JL, Carver GD, Johnson PA, Perry KL, Thompson JR. A rapid, sensitive and
341 inexpensive method for detection of grapevine red blotch virus without tissue extraction
342 using loop-mediated isothermal amplification. Arch Virol. 2019;164: 1453-1457.
343 19. Yan H, Zhang J, Ma D, Yin J. qPCR and loop mediated isothermal amplification for rapid
344 detection of *Ustilago tritici*. PeerJ. 2019;7: e7766.
345 20. Zhang L, Gleason C. Loop-mediated isothermal amplification for the diagnostic detection of
346 *Meloidogyne chitwoodi* and *M. fallax*. Plant Dis. 2019;103: 12-18.

347 21. Liu H, Wu W, Tan J, Li Y, Mi W, Jiang L. et al. Development and evaluation of a one-step
348 reverse transcription loop-mediated isothermal amplification for detection of *Citrus leaf*
349 *blotch virus*. J Virol Methods. 2019;270: 150-152.
350 22. Tahzima R, Foucart Y, Peusens G, Belien T, Massart S, De Jonghe K. New sensitive and
351 fast detection of *Little cherry virus 1* using loop-mediated isothermal amplification (RT-
352 LAMP). J Virol Methods. 2019;265: 91-98.
353 23. Raigond B, Verma A, Roach S, Kochhar T, Arjunan J, Kumar R, et al. One-step reverse
354 transcription loop-mediated isothermal amplification: a simple, sensitive and rapid assay for
355 detection of *Potato virus X* in potato leaves and tubers. Indian Phytopathol. 2019;72: 321-
356 328.
357 24. Wang KL, Deng QQ, Chen JW, Shen WK. Development of a reverse transcription loop-
358 mediated isothermal amplification assay for rapid and visual detection of *Sugarcane streak*
359 *mosaic virus* in sugarcane. Crop Protect. 2019;119: 38-45.
360 25. Tiberini A, Tomlinson J, Micali G, Fontana A, Albanese G, Tomassoli L. Development of a
361 reverse transcription-loop-mediated isothermal amplification (LAMP) assay for the rapid
362 detection of *Onion yellow dwarf virus*. J Virol Methods. 2019;271: 113680.
363 26. Raigond B, Verma A, Jandrajupalli S, Kochhar T, Sharma S, Chakrabarti SK. Squash print
364 reverse transcription loop-mediated isothermal amplification assay for detection of *Potato*
365 *leafroll virus* in single aphid and in potato. Potato Res. 2019: 1-4.
366 27. Wang Z, Spadoro J. Determination of target copy number of quantitative standards used in
367 PCR-based diagnostic assays. In: Ferr F, editor. Gene Quantification. 1998. Boston:
368 Birkhäuser; pp. 31-43.

369 28. Forootan A, Sjöback R, Björkman J, Sjögreen B, Linz L, Kubista M. Methods to determine
370 limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol
371 Detect Quantif. 2017;12: 1-6.
372 29. Zwart MP, Daròs JA, Elena SF. One is enough: in vivo effective population size is dose-
373 dependent for a plant RNA virus. PLoS Pathog. 2011;7: e1002122.
374
375
376

377 **Table 1.** Primers used in this study

378

Name	Sequence	Size (bp)	Reaction	Reference
F3	TTGGAGTCTTAGATGTTGCG	279		
B3	GGACACCGTCAACTAGGA			
FIP(F1c+F2)	CCTTCTCCAACTGTCGCAAGTCAC ATGCTAGGAAGTACCAC	n/a	LAMP	This study
BIP(B1c+B2)	CCGTGAGTTCTGAGTCAATGGTTG AGGCTCACCATCTCTTAA			
LoopF	CTCCATGCTCATCATACTCCAA	n/a		
LoopB	GCTCAGAACACTGAGGAGATT			
ToBRFV-F	GAAGTCCCGATGTCTGTAAGG	842	PCR & rt-PCR	[6]
ToBRFV-R	GTGCCTACGGATGTGTATGA			
ToB5520F	GTAAGGCTTGCAAAATTTCGTTCG	79	qPCR	[14]
ToB5598R	CTTTGGTTTTTGTCTGGTTTCGG			
qF1	CGGTGGATATGCCAGTGCTTG	99	qPCR	This study
qR1	TGTCCGACTCCTTGAGCACAG			

379

380

381

382 **Figure legends**

383 **Fig. 1.** Alignment of partial genomic sequences of selected ToBRFV, TMV and ToMV strains.
384 Locations of LAMP primers were highlighted and the primer name indicated above. Degenerated
385 nucleotides within the primer binding sites were bolded. Italic letters indicate the sequences
386 present in gBlock$^{\Delta F1c}$ and gBlock$^{\Delta B2}$ that differentiate the two gBlocks from gBlock^{ToBRFV}. Line
387 1, represents five ToRBFV strains. Lines 2 and 3, are sequences from two ToBRFV strains.
388 Lines 4-6 are sequences from three TMV strains. Lines 7 and 8 are sequences from two ToMV
389 strains. (Detailed description of the virus strains is list in Table S1).

390

391 **Fig. 2.** LAMP (A-C), PCR (D) and qPCR (E) tests on gBlocks. A, LAMP test on serial dilutions
392 of gBlock^{TMV}, gBlock^{ToMV} and gBlock^{ToBRFV}. B, LAMP test on two dilutions of gBlock$^{\Delta F1c}$ and
393 gBlock$^{\Delta B2}$. C, Repeated LAMP tests on gBlock^{ToBRFV} at the concentration of 6 molecules per
394 reaction. 40 repeats were conducted but only eight were shown; 35 out of the 40 were positive. D,
395 PCR test on serial dilutions of gBlock^{ToBRFV} using the primer pair ToBRFV-F/ToBRFV-R. M,
396 GeneRuler Express DNA Ladder. E, qPCR test on serial dilutions of gBlock^{ToBRFV} using the
397 primer pair qF1/qR1.

398

399 **Fig. 3.** LAMP (A and C) and rt-PCR (B and D) tests on total RNA extracted from five tomato
400 samples infected by ToBRFV. A and B, Tests on five original RNA samples. a, from tomato fruit;
401 b, from tomato leaf; c-e, from tomato flesh. M, GeneRuler express DNA ladder. C and D, Tests
402 on serial dilutions (1-8) of a RNA sample pooled from same volumes of each of the five original
403 RNA samples. M', TrackIt 100 bp DNA ladder.

404

405 **Fig. 4.** LAMP (A), PCR (B) and qPCR (C) tests on serial dilutions (1-7) of cDNA synthesized
406 from a RNA sample pooled from same volumes of each of the five original RNA samples. M,
407 GeneRuler express DNA ladder. Primer pair used in PCR was ToBRFV-F/ToBRFV-R and those
408 used in qPCR were qF1/qR1 for Test 1 and ToB5520F/ToB5598R for Test 2.
409

F3

```
1 TAGATCTCGAAACCCGTCAGAAGTTTGGAGTCTTAGATGTTGCGACCAAAAGATGGTTAAT
2 TAGATCTCGAAACCCGTCAGAAGTTTGGAGTCTTAGATGTTGCGACCAAAAGATGGTTAAT
3 TAGATCTCGAAACCCGTCAGAAGTTTGGAGTCTTAGATGTTGCGACCAAAAGATGGTTAAT
4 TAGATCTCGAAACCCGTCAAAAGTTCGGAGTTCTGGATGTGGCATCTAGGAAGTGGTTGAT
5 TTGACCTTGAAACCCGTCAAAAGTTTGGAGTCTTGGATGTTGCATCTAGGAAGTGGTTGAT
6 TTGACCTTGAAACCCGTCAAAAGTTTGGAGTCTTGGATGTCGCATCTAGGAAGTGGTTAAT
7 TTGACCTTGAAACTCGTCAAAAGTTCGGAGTCTTGGATGTTGCTTCGAAAAGGTGGCTAGT
8 TTGACCTTGAAACTCGTCAAAAGTTCGGAGTTCTGGATGTTGCTTCGAAAAGGTGGCTAGT
```

F2

```
1 TAAACCTTTAGCCAAGAATCACGCATGGGGCGTTATTGAAACACATGCTAGGAAGTACCAC
2 TAAACCTTTAGCCAAGAATCACGCATGGGGCGTTATTGAAACACATGCTAGAAAGTACCAC
3 TAAACCTTTAGCCAAGAATCACGCATGGGGCGTTATTGAAACACATGCTAGGAAGTACCAC
4 TAAACCGACTGCTAAGAGTCACGCATGGGGCGTTGTTGAGACTCATGCGAGGAAATATCAC
5 CAAACCAACGGCCAAGAGTCATGCATGGGGTGTTGTCGAAACCCACGCGAGGAAGTATCAT
6 TAAACCAACGGCCAAGAGTCATGCATGGGGTGTTGTTGAAACCCACGCGAGGAAGTATCAT
7 TAAACCATCCGCAAAGAACCATGCATGGGGGGTTGTTGAGACTCATGCGAGGAAATATCAC
8 TAAACCATCCGCAAAGAACCATGCATGGGGGGTTGTTGAGACTCATGCGAGGAAATATCAC
```

LoopF F1c

```
1 GTTGCACTTTTGGAGTATGATGAGCATGGAGTGGTAACTTGCGACAGTTGGAGAAGGGTGG
2 GTTGCACTTTTGGAGTATGATGAGCATGGAGTGGTAACTTGCGACAGTTGGAGAAGGGTGG
3 GTTGCACTTTTGGAGTATGATGAGCATGGAGTGGTAACTTGCGACAGTTGGAGAAGGGTGG
4 GTTGCACTTCTGGAGTACGATGAGCAGGGTATAGTAACTTGCGACAACTGGAGAAGAGTTG
5 GTGGCGCTTTTGGAATATGATGAGCAGGGTGTGGTAACATGCGATGATTGGAGAAGAGTAG
6 GTGGCGCTTCTGGAATATGATGAGCAGGGTGTGGTGACATGCGATAATTGGAGAAGAGTAG
7 GTCGCATTACTGGAGCACGATGAATTTGGCATTATTACGTGCGATAACTGGCGACGGGTGG
8 GTCGCATTACTGGAGCACGATGAATTTGGCATTATTACGTGCGATAACTGGCGACGGGTGG
```

B1c LoopB

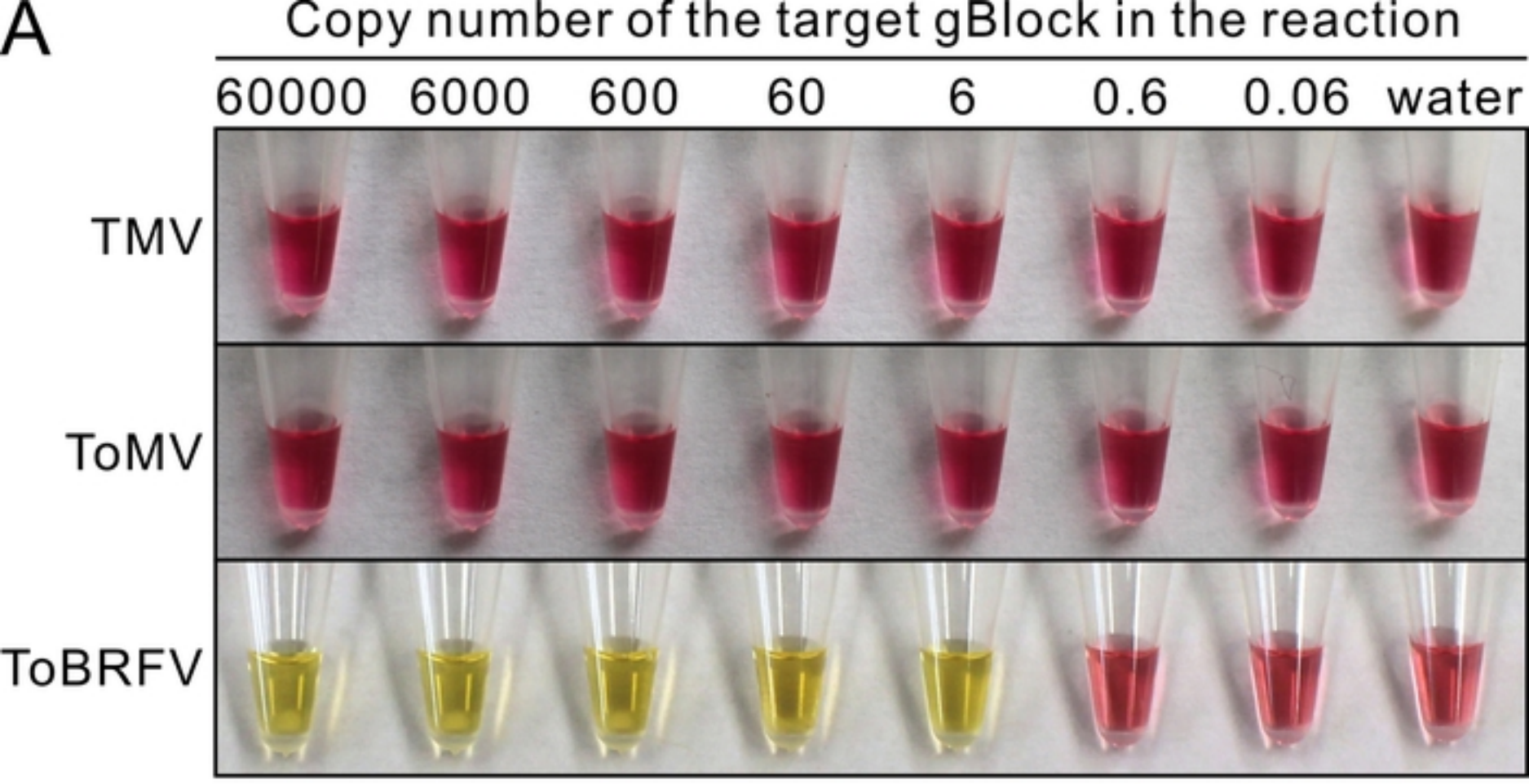
```
1 CCGTGAGTTCTGAGTCAATGGTTTATTCTGATATGGCAAAGCTCAGAACACTGAGGAGATT
2 CCGTGAGTTCTGAGTCAATGGTTTATTCTGATATGGCAAAGCTCAGAACACTGAGGAGATT
3 CCGTGAGTTCTGAGTCAATGGTTTATTCTGATATGGCAAAGCTCAGAACACTGAGGAGATT
4 CTGTTAGTTCTGAGTCGGTTGTATATTCTGATATGGCGAAACTCAGAACTCTGAGGAAATT
5 CTGTCAGCTCTGAGTCTGTTGTTTATTCCGACATGGCGAAACTCAGAACTCTGCGCAGACT
6 CTGTCAGCTCTGAGTCTGTTGTTTATTCCGACATGGCGAAACTCAGAACTCTGCGCAGACT
7 CTGTGAGTTCTGAGTCGGTAGTATATTCTGATATGGCTAAACTTAGGACTCTGAGAAGATT
8 CTGTGAGTTCTGAGTCGGTAGTATATTCTGATATGGCTAAACTCAGGACTCTGAGAAGATT
```

B2 B3

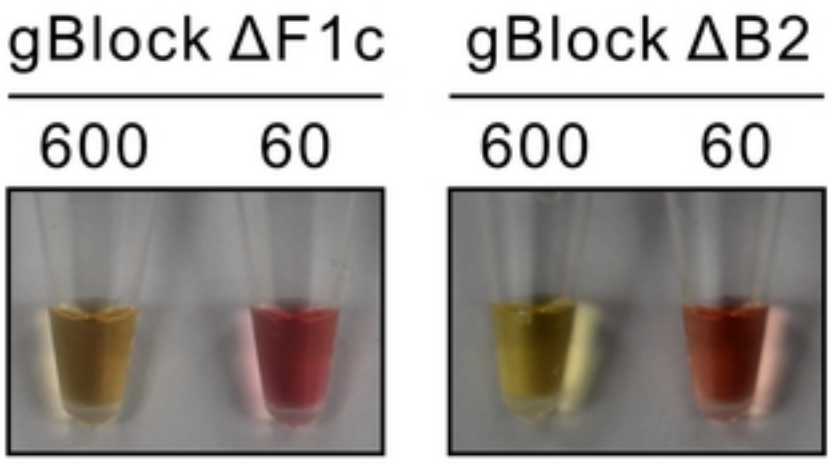
```
1 ATTAAGAGATGGTGAGCCTCATGTCAGCAGTGCTAAAGTCGTCCTAGTTGACGGTGTCCCG
2 ATTAAGAGATGGTGAGCCTCATGTCAGCAGTGCTAAAGTCGTCCTAGTTGACGGTGTCCCG
3 ATTAAGAGATGGAGAGCCTCATGTCAGCAGTGCTAAAGTCGTCCTAGTTGACGGTGTCCCG
4 GCTTCAAAATGGTGAACCTCATGTTAGTAACGCAAAGGTTGTTCTCGTGGACGGTGTCCCA
5 GCTTCGAAACGGTGAACCGCATGTCAGTAGCGCAAAGGTTGTTCTTGTGGACGGAGTTCCG
6 GCTTCGAAACGGAGAACCGCATGTCAGTAGCGCAAAGGTTGTTCTTGTGGACGGAGTTCCG
7 GCTCAAAGATGGAGAACCACACGTTAGTTCAGCAAAGGTTGTTTTGGTGGATGGCATTCCA
8 GCTTAAAGATGGAGAACCACACGTTAGTTCAGCAAAGGTGGTTTTGGTGGATGGCGTTCCA
```

Fig 1

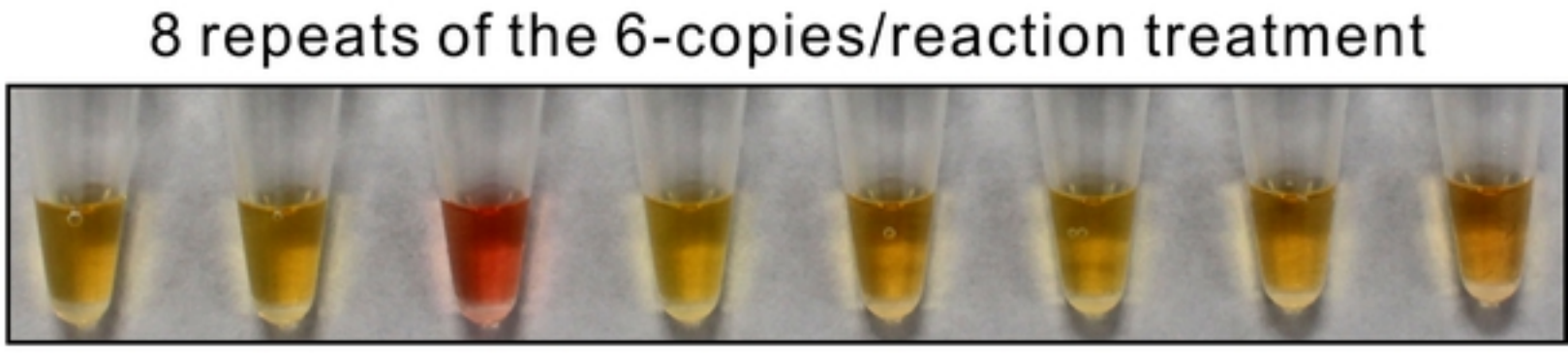
A



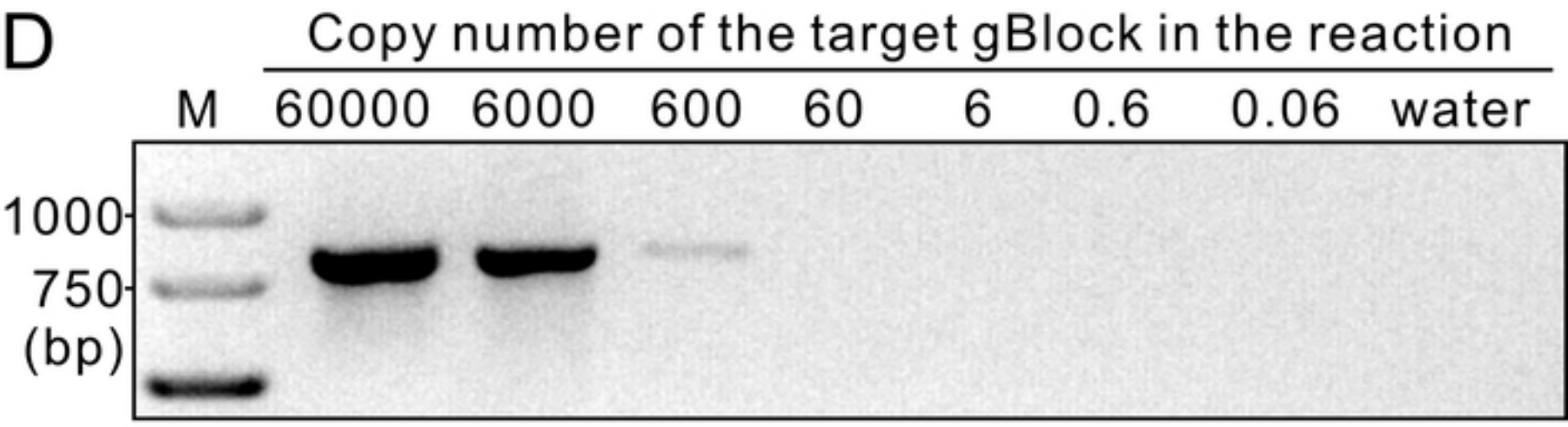
B



C



D



E

	Copy number of the target gBlock in the reaction							
	60000	6000	600	60	6	0.6	0.06	water
Cq (mean±SD)	21.86 ±0.02	25.26 ±0.03	28.65 ±0.12	32.10 ±0.19	35.09 ±0.36	38.98	n/a	n/a
#repeats with a Cq	3	3	3	3	3	1	0	0

Fig 2

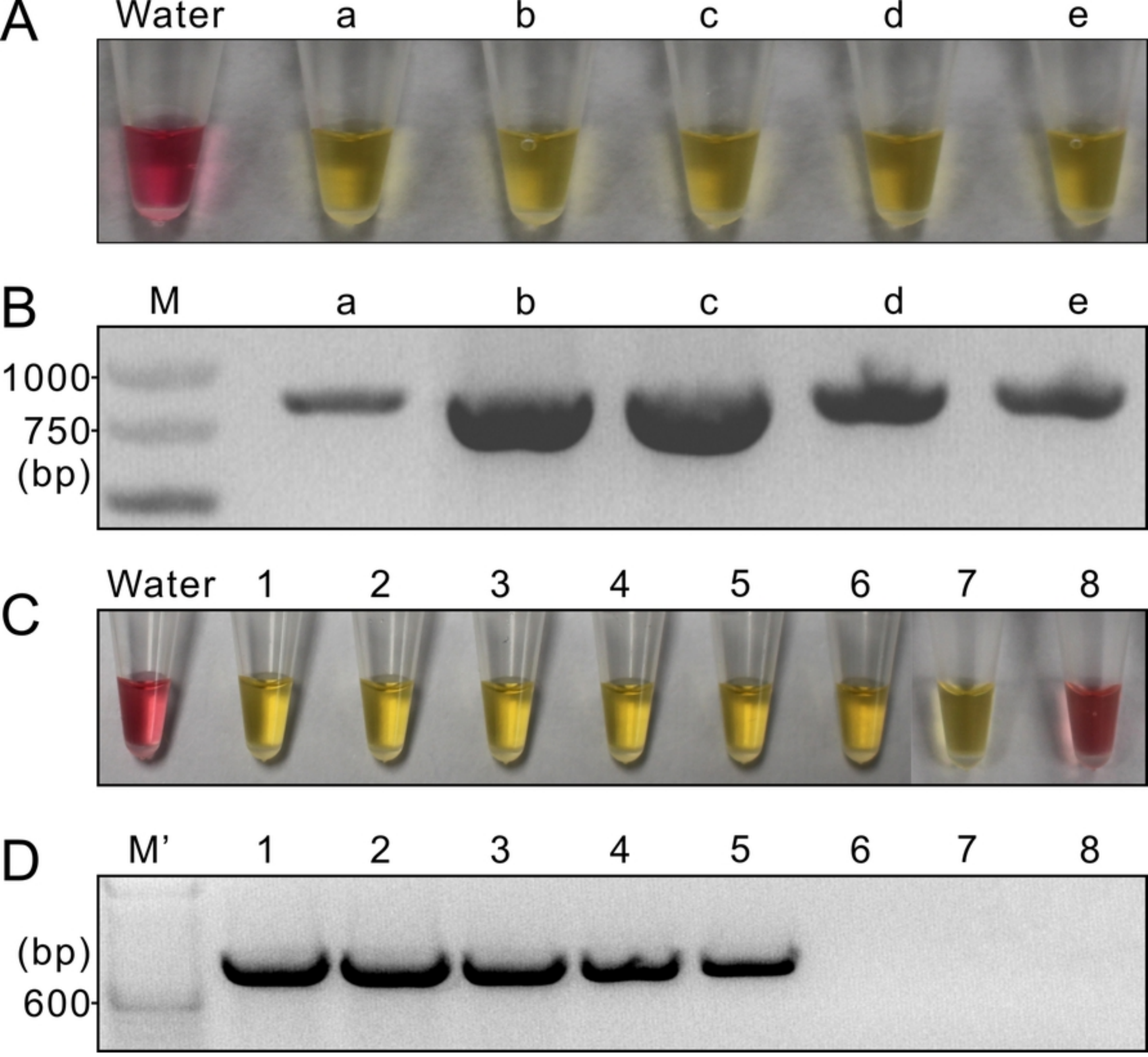
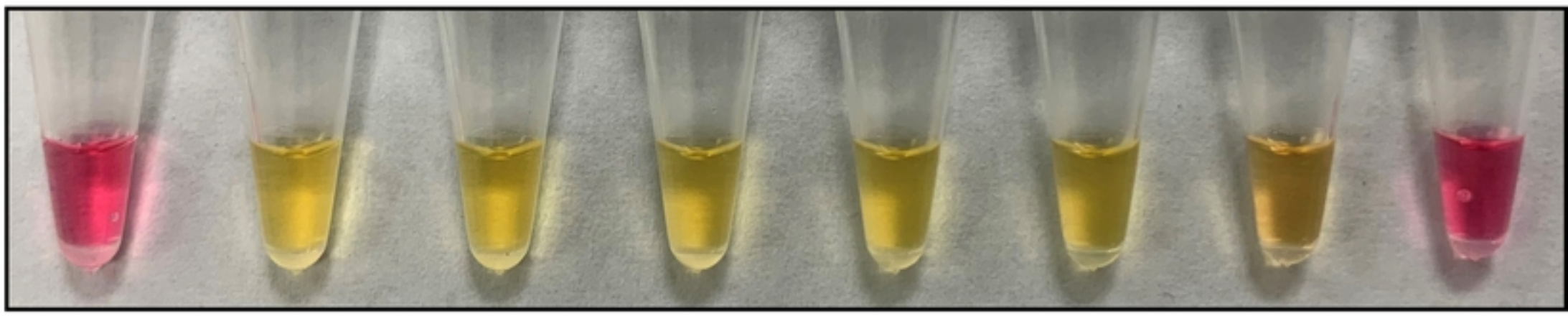
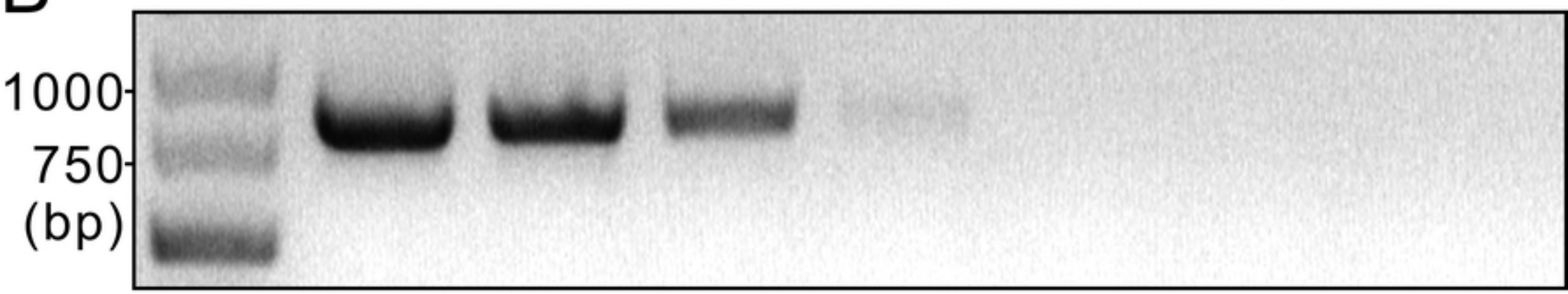

A
Water
a
b
c
d
e
B
M
a
b
c
d
e
1000
750
(bp)
C
Water
1
2
3
4
5
6
7
8
D
M'
1
2
3
4
5
6
7
8
(bp)
600

Fig 3

A



B



C

		1	2	3	4	5	6	7
Test1	Cq (mean±SD)	19.14 ±0.04	22.64 ±0.11	26.03 ±0.02	29.61 ±0.06	33.38 ±0.24	36.95 ±0.51	n/a
	#repeats with a Cq	3	3	3	3	3	2	0
Test2	Cq (mean±SD)	20.22 ±0.14	23.55 ±0.10	26.83 ±0.05	30.40 ±0.29	33.88 ±0.55	35.85 ±0.38	n/a
	#repeats with a Cq	3	3	3	3	3	3	0

Fig 4