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## Versatile and flexible microfluidic qPCR test for high-throughput SARS-CoV-2 and cellular response detection in nasopharyngeal swab samples

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### ► To cite this version:

Julien Fassy, Caroline Lacoux, Sylvie Leroy, Latifa Noussair, Sylvain Hubac, et al.. Versatile and flexible microfluidic qPCR test for high-throughput SARS-CoV-2 and cellular response detection in nasopharyngeal swab samples. 2020. hal-03052294

**HAL Id: hal-03052294**

**<https://hal.science/hal-03052294v1>**

Preprint submitted on 10 Dec 2020

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1 **Versatile and flexible microfluidic qPCR test for high-throughput SARS-CoV-2 and cellular**  
2 **response detection in nasopharyngeal swab samples**  
3

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23

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30

31 **Abstract**

32 The emergence and quick spread of SARS-CoV-2 has pointed at a low capacity response for testing  
33 large populations in many countries, in line of material, technical and staff limitations. The traditional  
34 RT-qPCR diagnostic test remains the reference method and is by far the most widely used test. These  
35 assays are limited to a couple of probe sets, require large sample PCR reaction volumes, along with  
36 an expensive and time-consuming RNA extraction steps. Here we describe a quantitative nanofluidic  
37 assay that overcomes some of these shortcomings, based on the Biomark instrument from Fluidigm.  
38 This system offers the possibility of performing 4608 qPCR end-points in a single run, equivalent to  
39 192 clinical samples combined with 12 pairs of primers/probe sets in duplicate, thus allowing the  
40 monitoring in addition to SARS-CoV-2 probes of other pathogens and/or host cellular responses  
41 (virus receptors, response markers, microRNAs). Its 10 nL range volume is compatible with sensitive  
42 and reproducible reactions that can be easily and cost-effectively adapted to various RT-qPCR  
43 configurations and sets of primers/probe. Finally, we also evaluated the use of inactivating lysis  
44 buffers composed of various detergents in the presence or absence of proteinase K to assess the  
45 compatibility of these buffers with a direct reverse transcription enzymatic step and we propose  
46 several procedures, bypassing the need for RNA purification. We advocate that the combined  
47 utilization of an optimized processing buffer and a high-throughput real-time PCR device would  
48 contribute to improve the turn-around-time to deliver the test results to patients and increase the  
49 SARS-CoV-2 testing capacities.

50

51

52 **Introduction**

53 To control the pandemic and monitor virus propagation of SARS-CoV-2 in real time, extensive testing  
54 is necessary. Although alternatives are available (1, 2), viral load detection from nasopharyngeal or  
55 saliva samples is still the most appropriate method to identify SARS-CoV-2 carriers. The current  
56 diagnostic testing methods recommended by the Centers for Diseases Control (CDC) and the World  
57 Health Organization (WHO) are based on a traditional RT-qPCR assay, with validated primers (3).  
58 However, the availability of this assay has been a major hurdle in the orderly and efficient  
59 management of the pandemic because of reagent shortages, as well as material and staff limitations.  
60 A rapid processing of the samples is also a crucial factor. To further stem the spread of coronavirus  
61 disease 2019 (COVID-19), a series of innovative approaches have been recently proposed (4).  
62 Notably, to face material shortage and reduce processing times, two kinds of process optimization  
63 should be undertaken: (1) multiplexing sample and PCR probes, as well as (2) reducing the steps in  
64 sample preparation.

65 Most approved RT-qPCR assays are “one-step” kits that should be performed on standard real-time  
66 thermocyclers. When sample pooling (5) is not used, throughput is quite limited and the use of  
67 alternative systems, such as the Biomark™ HD device from Fluidigm may overcome this limitation.  
68 The Biomark™ HD device is a nanofluidic automated real-time PCR system that exploits the  
69 microfluidic technology through the use of dynamic arrays of integrated fluidic circuits (IFCs). Using,  
70 for example, the 192.24 Integrated Fluid Circuit (IFC), 192 samples can be processed in parallel with  
71 24 independent sets of probes, allowing increased confidence in viral RNA detection as additional  
72 SARS-CoV-2 probes can be included. Probes to detect supplementary targets such as other RNA  
73 viruses, or host response genes can also be included. This flexibility presents a clear added value for  
74 both clinical monitoring of viral and bacterial pathogens as well as for research studies (6, 7). Another  
75 advantage of this system resides in the low amount of reagents needed compared to classical real-  
76 time PCR machines. The reaction volume is down to the 10 nL-scale compared to the 10 µL scale in  
77 classical qPCR, thus reducing reagent requirement, an important point in times of shortages.

78 The second improvement resides in the reduction of the number of steps for sample preparation. RT-  
79 qPCR detection starts with viral RNA extraction. This is time consuming for laboratories that are not  
80 equipped with high-throughput automated systems. It is also reagent consuming, and as March  
81 2020, laboratories have suffered from major RNA extraction kit shortages. Several teams have  
82 proposed RNA extraction-free protocols. The resulting improvement in processing times were  
83 balanced by a loss in sensitivity. The comparison of these different studies is made difficult by the  
84 great variabilities of the protocols. This includes parameters such as the type of collection media, the  
85 use of additives such as detergents, heat-inactivation, or selection of a particular RT-qPCR kit (8–16).  
86 In 2 of these studies, measurements on a large set of clinical samples demonstrated the effectiveness  
87 of direct RT-PCR assays with or without heat inactivation on various transport medium (VTM) (8, 9).  
88 Srivatsan et al. proposed to collect dry swabs and elute them in Tris-EDTA (TE) to eliminate collection  
89 medium dilution and variation, and to bypass the RNA extraction step (17). This simplified protocol  
90 was as sensitive as a conventional protocol (sample collection in VTM). They also evaluated the  
91 addition of detergents (IGEPAL, Triton X-100 and Tween-20) that facilitates virus inactivation in the  
92 elution medium. Addition of all three detergents resulted in a loss of sensitivity.

93 In the present study, we evaluated the use of a high-throughput real-time PCR device, the Biomark™  
94 HD, to increase throughput, flexibility in probe inclusion, and decrease reagent consumption,  
95 together with an optimized protocol for SARS-CoV-2 RNA detection without RNA extraction.

96

97 **Materials and methods**

98 **Primers and probes for the detection of viral and cellular genes:**

99 All the DNA primers and probes used in this study are listed and described in Table 1 and 2.

100 **Positive control plasmid:**

101 Positive control plasmid containing the full length nucleocapsid coding sequence (N) was purchased  
102 at Integrated DNA Technologies (2019-nCoV\_N\_Positive Control; cat. no. 10006625). Plasmid was  
103 supplied at a final concentration of 200 000 copies/ $\mu$ L in IDTE pH=8 buffer, and has been used to  
104 evaluate both the detection efficiency and sensitivity of the Biomark HD System Analysis.

105 ***In vitro* Transcription:**

106 The synthetic transcript for the 2019-nCoV N coding sequence (N transcript) was generated using the  
107 2019-nCoV\_N\_Positive Control plasmid. The template for the *in vitro* transcription was produced by  
108 PCR: briefly, 6 ng of plasmid was used to amplify the entire coding sequence of the N gene using: T7-  
109 For-sens (ATATAATACGACTCACTATAGGATGTCTGATAATGGACCC; T7 promoter sequence underlined)  
110 and Rev-sens (TTAGGCCTGAGTTGAGTC) as primers and the Q5-High Fidelity DNA polymerase (New  
111 England Biolabs, cat. no. M0491S). Thermal cycling conditions were: 98°C for 30 s, 40 cycles of 98°C  
112 for 10 s, 52°C for 30 s, 72°C for 1 min followed by 72°C for 2 min (SimpliAmp Thermal Cycler,  
113 ThermoFisher Scientific). The size of the PCR product was verified on a 1.5% agarose gel in 0.5X TAE  
114 and further purified using the QIAquick PCR purification kit (Qiagen, cat. 28106). Template was used  
115 in the *in vitro* transcription reaction using the T7 RiboMAXExpress Large Scale Production System  
116 (Promega, cat. no. P1320) according to manufacturer's instructions. The *in vitro* transcript was  
117 extracted using phenol chloroform isoamyl followed by a chloroform wash and further precipitated  
118 using 300 mM sodium acetate in absolute ethanol at -20°C, over-night. Sample was centrifugated at  
119 4°C for 30 min and washed twice with 70% cold ethanol. The RNA pellet was dried at RT for 5 min  
120 and resuspended in 100  $\mu$ L water.

121 **Clinical samples:**

122 *Pulmonology department, Nice University Hospital*: 20 clinical samples from study participants were  
123 collected as part of the ELISpot study (ClinicalTrials.gov Identifier: NCT04418206). Participants were  
124 enrolled after signing written informed consent. Ethics committee approval was obtained from the  
125 “Comité de Protection des Personnes Sud Méditerranée V” (registration # 2020-AO1050-39) on April  
126 22, 2020. Nasopharyngeal swabs were collected in ESwab™ (COPAN) transport medium (2 mL),  
127 stored at 4°C between sample collection and transport to the laboratory. One COVID-19 confirmed  
128 patient was collected consecutively using 2 different transport media: ESwab™ or a Tris-EDTA (TE: 10  
129 mM Tris HCl pH 7.0, 2 mM EDTA, 20 µg/mL yeast tRNA) buffer.

130 *Garches Hospital (AP-HP)*: Purified RNA samples (n=92) from nasopharyngeal swabs with known  
131 COVID-19 status were obtained from the Garches Hospital following an ISO 15189 certified lab  
132 protocol previously described (18) and used to validate the BIOMARK™ HD protocol. Additional  
133 frozen VTMs from 55 patients with known COVID-19 status collected in 1-2 mL of saline buffer were  
134 obtained to set up the protocol. All samples were recorded for traceability on the basis of a unique  
135 barcode identifier.

136 *Bioesterel laboratories*: Samples (18 in virucide VTM, 2 in non-virucide VTM, Lingen) were stored  
137 overnight at 4°C and processed the next day. A generic consent form was signed by the patients,  
138 allowing the utilization of the samples for scientific research. All samples were anonymized and their  
139 COVID-19 status was recorded.

#### 140 **RNA extraction:**

141 Both miRNeasy Serum/Plasma Advanced Kit (Qiagen, cat. no. 217204) and QIAamp Viral RNA Mini Kit  
142 (Qiagen, cat. no. 52906) were used for Total RNA extraction from clinical samples according to  
143 manufacturer's instructions and using the Qiacube (Qiagen) apparatus. Final elution was performed  
144 in 20 µL water and 60 µL of AVE buffer for the miRNeasy Serum/Plasma Advanced Kit and QIAamp  
145 Viral RNA Mini Kit, respectively. Total RNA (containing miRNAs) were directly used for further  
146 analysis or stored at -20°C.

#### 147 **Detergent treatments of clinical samples:**

148 All clinical samples were handled in a biosafety level 2 laboratory and under biological safety cabinet  
149 using the adapted biosafety personal protective and respiratory equipment, according to the  
150 recommendations of the French society of microbiology (<https://www.sfm-microbiologie.org/>). The  
151 following detergents were used: Triton X-100 (Sigma, cat. no. T9284-100ML), Brij™ 35 (Millipore, cat.  
152 No. 1.01894.0100), Tween-20 (Sigma, cat. no. P7949-500ML), Brij™ O10 (Sigma, cat. no. P6136-100G)  
153 and Poly Ethylene Glyco-600 (Aldrich, cat no. 202401-250G). They were included alone or in  
154 combination to prepare a 2X detergent master mix that was added to clinical transport media  
155 samples. A 10 mg/mL Proteinase K (Sigma P4850) solution was mixed extemporaneously with the 2X  
156 detergent master mix to reach a 2 mg/mL final concentration. Heating treatments were carried out  
157 at 95°C for 5 min in a thermo-cycler apparatus (SimpliAmp Thermal Cycler, ThermoFisher Scientific).  
158 Treatment using the Quick Extract™ DNA Extraction Solution (Lucigen, QE09050) was performed  
159 using the same volume of reagent and sample, according to the manufacturer's instructions.  
160 Mixtures were then incubated at 95°C for 5 min or at 60°C for 10 min in a thermo-cycler. TE buffer  
161 was prepared using 10 mM Tris-HCl pH7.0, 2 mM EDTA, 20 µg/mL yeast tRNA with or without 0.5%  
162 Triton X-100.

### 163 **Detection of mRNA expression protocol using the Biomark HD System Analysis**

#### 164 *Reverse Transcription using the Fluidigm Reverse transcriptase:*

165 Extracted RNA (2 µL) or processed clinical samples were reverse transcribed using the Reverse  
166 Transcription Master Mix kit according to the manufacturer's instructions (catalog number # PN 100-  
167 6297). Thermal cycling conditions were: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min.

#### 168 *Reverse transcription using the Vilo SuperScript IV step:*

169 2.5 µL of processed clinical samples were used in a reverse transcription reaction using the  
170 SuperScript IV Vilo Master Mix (ThermoFisher Scientific, cat. no. 11756500) following the  
171 manufacturer's instructions. Reactions were incubated at 25°C for 10 min, 55°C for 10 min, 85°C for 5  
172 min

#### 173 *cDNA pre-amplification step:*



174 2.5  $\mu\text{L}$  of cDNA were preamplified using the Preamp Master Mix kit (Fluidigm, cat. no. PN 100-5744)  
175 according to the manufacturer's instructions: 1  $\mu\text{L}$  of Preamp Master Mix was combined with 1.25  $\mu\text{L}$   
176 of Pooled Taqman assay mix and 2.5  $\mu\text{L}$  of cDNA in a 5  $\mu\text{L}$  total volume reaction. The Pooled Taqman  
177 assay (180 nM) used in these reactions was prepared from an intermediate pooled Taqman assay  
178 solution (6.7  $\mu\text{M}$  Forward and Reverse primers, 1.7  $\mu\text{M}$  probes). Thermal cycling conditions were:  
179 95°C for 2 min followed by 20 cycles of 95°C for 15 s, 60°C for 2 min. After each preamplification  
180 reaction, samples were diluted 1:5 by adding nuclease-free water up to 25  $\mu\text{L}$ .

181 *One step "RT-Preamplification":*

182 One step "RT-Preamplification reactions" were performed using the Cells Direct One-Step qRT-PCR  
183 kit (Invitrogen, cat. no. 46-7200) according to a protocol adapted from the Reverse Transcription-  
184 Specific Target Amplification (Fluidigm). Briefly 5.5  $\mu\text{L}$  of total RNA were mixed with 6.25  $\mu\text{L}$  the  
185 Pooled Taqman assay, 0.5  $\mu\text{L}$  Superscript III RT/ Platinum Taq, 12.5  $\mu\text{L}$  2x Reaction mix and nuclease-  
186 free water to 25  $\mu\text{L}$  total volume. Thermal cycling conditions were: 42°C for 15 min, 95°C for 2 min  
187 followed by 15 to 20 cycles of 95°C for 15 s, 60°C for 2 min. Then, samples were diluted 1:5 using  
188 nuclease-free water up to 125  $\mu\text{L}$ .

189 *Real time qPCR using Biomark HD System:*

190 PCR was performed following Gene Expression Standard TaqMan Assays protocol (Fluidigm cat n°  
191 100-6170 C1), using a 10X assays mix and a pre-sample mix prepared separately. The 10x assays mix  
192 was prepared by mixing 2  $\mu\text{L}$  of combined Primer (Forward/Reverse 6.7  $\mu\text{M}$ , Probe 1.7  $\mu\text{M}$ ) and 2  $\mu\text{L}$  2X  
193 Assay Loading Reagent (Fluidigm PN 100-7611) to a final volume of 4  $\mu\text{L}$  (per reaction). The pre-  
194 sample mix was prepared by mixing 2  $\mu\text{L}$  TaqMan Universal PCR Master Mix (2X) (Life Technologies  
195 PN 4304437) and 0.2  $\mu\text{L}$  20X GE Sample Loading Reagent (Fluidigm PN 100-7610) and 1.8  $\mu\text{L}$   
196 preamplified cDNA to a final volume of 4  $\mu\text{L}$ .

197 Then, 3  $\mu\text{L}$  of 10x assays mix and of pre-sample mix are transferred into the 192.24 IFC, loaded into  
198 the Biomark IFC controller RX and transferred to the Biomark HD apparatus. Thermal cycling

199 conditions were as follows: 50°C for 120 s, 95°C for 600 s followed by 20 cycles of 95°C for 15 s, 60°C  
200 for 1min.

#### 201 **Detection of microRNA-expression protocol using the Biomark HD System Analysis**

202 RNAs (1 µL) were processed using the miScript 2 RT kit (QIAGEN cat n° 218161). Preamplification was  
203 performed using 5 µL of diluted cDNA using the mi Script microfluidics PreAMP kit (QIAGEN, cat n°  
204 331455) and following the 384-plex preamplification cycling conditions (see manufacturer's  
205 protocol). QPCR was performed following miScript microfluidics PCR kit (QIAGEN cat n° 331431) with  
206 commercially-available primers (miScript Primer assays). Six µL of assays mix and sample mix were  
207 prepared as described in the manufacturer's protocol and 5 µL of each are loaded onto the 96.96 IFC  
208 plates (Fluidigm cat n° BMK-M-96.96). The list of microRNAs (miRNAs) tested in this study is  
209 presented in Table S1.

#### 210 **Biomark HD System Analysis:**

211 Exhaustive protocols are available at:

212 <https://dx.doi.org/10.17504/protocols.io.bd3ii8ke> (Two-Step qPCR Protocol)

213 <https://dx.doi.org/10.17504/protocols.io.bnx4mfgw> (One-Step qPCR Protocol)

#### 214 **Fluidigm HD System qPCR Analysis:**

215 qPCR results were analyzed using the Real Time PCR analysis software provided by Fluidigm.

#### 216 **Statistical analysis**

217 Statistical analyses were performed using GraphPad Prism software. Results are given as mean ±  
218 SEM. Two-tailed unpaired Student's t-test was used for single comparisons and one-way ANOVA  
219 followed by Bonferroni post hoc test was used for multiple comparison. P-value < 0.05 was  
220 considered significant.

221

222

223 **Results**

224 *Performance and validation of a Biomark-HD SARS-CoV-2 qPCR assay*

225 We first set up a classical Fluidigm qPCR assay protocol that combines all primers/probe sets in a  
226 single reaction. This system requires a pre-amplification step after cDNA synthesis. This is due to the  
227 low volume of the IFC reaction chamber (9nl). As a result and compared to the traditional RTqPCR  
228 protocol (cDNA synthesis and qPCR), three consecutive reaction steps (RT, pre-amplification and  
229 qPCR) are required in the classical Biomark-HD assay.

230 To evaluate the efficiency of the qPCR, we first assessed the test sensitivity using a range of synthetic  
231 viral N transcript dilutions with the US CDC primers/probe set N1. We found that the N transcript  
232 detection limit was seven transcript copies per reaction (Fig.1A). Linear regression showed a good  
233 correlation across Cq value and dilution series of N transcript ( $R^2=0.9846$ ) (Fig.1A). Similar sensitivity  
234 experiments were performed with additional primers/probes from the US CDC and by diluting the  
235 transcript in TE buffer without (Fig. S1A) or with (Fig. S1B) a RNA purification step. Dilution of a SARS-  
236 CoV-2 positive clinical sample also indicated a large dynamic range of detection of 6 orders of  
237 magnitude for N1 and the E (19) primers/probe (Fig. 1B). We then tested the combination of various  
238 primers/probe sets during the pre-amplification reaction, showing that 2 distinct mixes (mix #1: N1,  
239 E, human RNP; mix #2: ORF1ab, E, N and human RNP) gave similar data on clinical samples (Fig. 1C).  
240 We finally evaluated the performance of the test on a set of 18 samples from positive patients  
241 showing a large range of Cq values. The initial reference analysis was performed using the Elitech  
242 Genefinder™ COVID-19 detection kit, comprising 3 sets of viral primers/probe targeting the same  
243 viral genes as the mix #2. We noticed excellent correlations between both methods for the detection  
244 of the 3 transcripts with  $R^2$  above 0.95 (Fig. 1D), only affected by some high-range samples (i.e. Cq  
245 below 5), which displayed a saturation on Biomark. Similarly, we also evaluated the performance of  
246 the test using the probe of the viral N gene on a set of 92 purified RNA samples of known status,  
247 comprising 15 positive samples, previously analyzed using the GeneFirst COVID-19 detection kit  
248 (comprising two sets of primers/probe including the N target). We plotted the correlation for the Cq

249 obtained for the detection of the N transcript using both methods, showing a  $R^2$  of 0.97, with no false  
250 positive detected on the remaining 77 negative samples (Fig. S2).

#### 251 *Optimization of the test*

252 Additional tests were performed to reduce handling and reaction times by optimizing cycle numbers,  
253 elongation times and primer concentrations in the preamplification reaction (Fig. S3). In another  
254 optimization, we combined RT and pre-amplification into a one-step reaction, using the Cells Direct  
255 One-Step qRT-PCR kit (ThermoFisher Scientific). We compared this method to the classical Fluidigm  
256 RT-qPCR on a set of 18 clinical samples with a wide range of Cq values. Very similar Cq values were  
257 obtained with both methods for the cellular (RNP) and for 2 viral (E and N) primers/probe sets. The  
258 detection limit for ORF1ab gene was even improved using the Cells Direct One-Step qRT-PCR kit-  
259 based protocol (Fig. 2A). Overall, this protocol offers the possibility to run 192 samples with 24  
260 couples of primers/probe sets (using the Fluidigm 192.24 IFC) corresponding to 4608 end-points in  
261 less than 3 hours.

#### 262 *Alternative RNA extraction method using the miRNeasy Serum/Plasma Advanced Kit (Qiagen)*

263 To limit the possible impact of a shortage of viral RNA extraction kits, we compared the performance  
264 of the QIAamp Viral mini kit to that of the miRNeasy Serum/Plasma Advanced Kit. A set of 18 SARS-  
265 CoV-2 positive samples with a wide range of Cq values was extracted using both protocols. The 36  
266 resulting RNA samples were run in parallel on the BIOMARK-HD using 3 human and 3 viral primers/  
267 probe sets. Identical Cq values were obtained for the human probe (RNP) with both extraction  
268 protocols. A slight gain of signal (around 1-2 Cq) was measured after QIAamp Viral purification for  
269 the 3 viral primer/probes (E, N and ORF1ab) (Fig. 2B). These data suggest that the QIAamp Viral RNA  
270 mini Kit slightly increases the efficiency of viral transcript extraction, but the miRNeasy  
271 Serum/Plasma Advanced Kit represents an acceptable alternative solution in case of shortage.

#### 272 *Use of the BIOMARK™ HD-based protocol to analyze the host Response to SARS-CoV-2 infection at* 273 *the mRNA and miRNA levels*

274 Exploiting the potential for multiplexing of the BIOMARK™ HD-based technology, we included sets of  
275 human primers/probe to evaluate the expression of genes required for viral entry (*ACE2*, *TMPRSS2*),  
276 as well as inflammatory / antiviral response genes (*CXCL8*, *IL1A*, *IL1B*, *IL6*, *IFNB1*, *IFIT1*). A set of  
277 miRNAs (see Supplemental Table 1 for detail) was also tested (Fig. 3A). We divided the patient  
278 samples (n=72) into groups, according to their viral load (strong, medium, weak or negative). *ACE2*  
279 and *TMPRSS2* were detected in all samples, regardless of the viral status, with Cq values close to  
280 those for RNP, suggesting they could represent valid human control genes to assess the quality and  
281 the presence of epithelial cells in the samples (Fig. 3B-C). The signal obtained for the inflammation  
282 and interferon responses was more heterogeneous (Fig. 3B). We particularly noticed a strong signal  
283 (in the range of 5 Cq) for *IFIT1* in the 3 samples with the highest viral load (Cq values < 10 for the viral  
284 probes) (Fig. 3C). Additionally, we evaluated the possibility to quantify miRNA levels in the same  
285 samples. A large number of miRNAs were detected in most of the samples (examples in Fig. 3B-C).  
286 Overall, our results demonstrate that cellular markers can be easily quantified in nasopharyngeal  
287 swabs and may provide useful information to refine the diagnosis or prognosis of COVID-19 patients.

#### 288 *Direct qPCR detection of SARS-CoV-2 using inactivating lysis buffers-based protocols*

289 Direct SARS-CoV-2 diagnostic methods have been described, mainly performing RT-qPCR directly on  
290 crude or heat-inactivated subject samples (8–10). An additional refinement would be to improve the  
291 sensitivity and safety of these protocols by the use of RT-qPCR-compatible inactivating lysis buffers  
292 containing detergents such as Triton X-100, a standard non-ionic detergent widely used for  
293 inactivation of enveloped viruses such as SARS-CoV (20, 21). We first compared a regular extraction-  
294 based method with a direct RT-qPCR protocol on a synthetic N transcript spike in TE buffer in the  
295 presence or the absence of 0.5% Triton X-100 and confirmed that Triton X-100 did not interfere with  
296 the efficiency of the RT-qPCR steps (Fig. S4A). We then performed two consecutive nasopharyngeal  
297 swab samplings on a COVID-19 diagnosed patient, using either a regular VTM or a TE buffer  
298 containing 0.5% Triton X-100 followed or not by a heating step at 65°C for 10 min. As expected, the  
299 presence of Triton X-100 and the heating process did not affect the detection of the human RNP

300 transcript for both RT-qPCR methods. Conversely, while the Triton X-100-lysis buffer / heating  
301 process slightly improved the signal for the virus N1 primers/probe using the regular extraction-  
302 based protocol, this treatment strongly inhibited the direct RT-qPCR method for the same cellular  
303 transcript (Fig. S4B), indicating that 0.5% Triton X-100-lysis buffers are not compatible for a sensitive  
304 direct SARS-CoV-2 RT-qPCR assay.

305 We then tested the use of additional detergents and emulsifiers (Tween-20, Brij™-35, Brij™ O10),  
306 alone or in combination with polyethylene glycol (PEG600), in the presence or the absence of a  
307 treatment with proteinase K (PK), followed by heat inactivation. The experiment was performed on 2  
308 clinical samples of known COVID-19 status, sampled in a commercial VTM medium, aliquoted and  
309 then treated in parallel using 16 distinct detergent conditions in the presence or absence of PK, for a  
310 total of 64 experimental conditions (Fig. 4 and S5). Additional controls included the crude and heat-  
311 inactivated samples, the use of Quick Extract™ DNA Extraction Solution (QE), which has been  
312 recently proposed as an alternative method to extraction (22, 23) as well as a regular extraction-  
313 based protocol (positive control). Three and five sets of SARS-CoV-2 (Fig. 4A) and human (Fig. 4B)  
314 primers/probe, respectively, were used in the same assay on a 192.24 dynamic array. In these  
315 conditions, a direct assay on a crude sample resulted in a 9 to 15-Cq increase, depending on the viral  
316 primers/probe set, when compared to the extraction method, with an even more pronounced effect  
317 when samples were heated 5 min at 95°C (Fig. 4A). While the addition of 0.5% Triton X-100 alone  
318 was similar to the crude direct assay condition, the combination of Triton X-100 with the emulsifier  
319 Brij™ O10 (Oleth-10) improved the detection for all viral primers/probes, with a drop of around 5 Cq  
320 compared to Triton X-100 alone. All other detergents used alone (Tween-20, Brij™ O10, Brij™-35)  
321 gave a similar signal as 0.5% Triton X-100 alone. Of note, the addition of PK resulted in an  
322 improvement of sensitivity in the presence of various detergents including Tween-20, Brij™ O10 and  
323 Brij™-35 while addition of PEG had no beneficial effect (Fig. 4 and Fig. S5).

324 Based on these results, we selected two of the best direct detergent based assays (Triton X-100 /  
325 Brij™ O10 and Tween-20 / PK) and compared the sensitivity of these methods with a crude direct

326 assay and the extraction method on additional clinical samples. For all direct assays, all samples were  
327 heat-inactivated at 95°C (5 min). Moreover, we used a set of 17 clinical samples from SARS-CoV-2-  
328 diagnosed patients collected in saline solution with a wide range of Cq values. To control pH  
329 conditions and limit RNA degradation, all samples were diluted in TE buffer. We plotted the  
330 relationships between Cq obtained for the detection of the N and ORF1ab primers/probe sets for the  
331 Biomark assay and the GeneFirst COVID-19 detection kit (Fig 5). As expected, an excellent correlation  
332 was obtained when comparing the RNA extraction-based Fluidigm protocol with the reference assay  
333 ( $R^2=0.8258$  and  $R^2=0.8208$ , all samples detected). Conversely to the data obtained with the  
334 commercial VTM, the best direct protocol corresponded to the crude assay (TE), approaching a very  
335 similar performance to that obtained with the extraction-based assay ( $R^2=0.6447$  and  $R^2=0.6782$ ,  
336 16/17 samples detected). The  $R^2$  values dropped in a dramatic way for the two direct detergents  
337 based assays. However, the detection sensitivity still appeared quite high with 11 to 14 positive  
338 samples detected. Overall, these data suggest that a direct qPCR method using saline as a VTM and a  
339 basic TE buffer followed by a 5 min inactivation step at 95°C can efficiently support detection of  
340 SARS-CoV2.  
341

342 **Discussion**

343 The present paper describes a reliable and flexible multiplex nanofluidic qPCR system-based protocol  
344 to detect SARS-CoV-2. Its versatile format makes it easily adaptable to multiple other pathogens and  
345 / or host cellular markers. The same run allows to test in parallel viral and host RNAs, including  
346 miRNAs. We show a high concordance between this method and clinically approved traditional qPCR  
347 tests. This assay addressed some of the challenges of RT-qPCR assays, including analyzing a larger  
348 number of reactions per run, making the assay more cost-effective and less time-consuming. Further,  
349 IFCs dynamic arrays not only reduces the reaction volume from about 10  $\mu$ L down to the 10 nL scale,  
350 but allows large multiplexing as well as increased parallelization throughput of qPCR reactions.

351 The flexibility of this platform may be used in biomarker studies aiming at predicting at diagnosis the  
352 severity (requirement or not for hospitalization/intensive care) or the length (development of so-  
353 called “long-COVID”) of the disease. Several studies have depicted an elevated innate and adaptive  
354 immune activation in severe COVID-19 patients (24–26), and a differential immune phenotype in  
355 moderate versus severe disease after the second week of infection (26, 27). The Biomark HD offers  
356 the possibility to extend the number of genes tested to biomarkers including pro-inflammatory  
357 cytokines, chemokines, interferons, tissue repair genes and miRNAs by nasopharyngeal swabs or in  
358 saliva.

359 An important limitation of current tests is the requirement for RNA extraction that constitutes an  
360 obstacle to scale-up the capacity of testing both in term of time and cost. Several groups have  
361 explored methods to circumvent RNA extraction by performing RT-qPCR directly on crude or heat-  
362 inactivated clinical samples (8–10). Overall these methods show that testing for SARS-CoV-2 infection  
363 can be performed without RNA extraction, with a limited loss in accuracy for determining negative  
364 and positive cases. While this procedure is simple and attractive, it might be improved by the  
365 addition of detergents to facilitate viral capsid lysis to release genomic RNA and also directly  
366 inactivate the virus to facilitate sample handling and safety. Several methods and commercial kits  
367 have developed approaches to lyse efficiently mammalian cells and directly perform RT-qPCR or



368 RNA-seq libraries. However, these methods are not fully optimized for virus lysis, which requires  
369 increased concentrations of detergents. Some studies indicate that Triton X-100, widely used in virus  
370 inactivation procedures, or Tween-20 may slightly improve or at least not interfere with the RT-qPCR  
371 SARS-CoV-2 direct testing of nasopharyngeal swabs or saliva (9, 16). However, these initial reports  
372 clearly mentioned that additional efforts were needed to optimize direct RT-qPCR assays on  
373 detergent-inactivated samples. We evaluated here the use of several detergents and emulsifiers  
374 (Triton X-100, Tween-20, Brij™-35, Brij™ O10), alone or in combination, in the presence or the  
375 absence of a treatment with PK or polyethylene glycol (PEG) followed by heat inactivation to assess  
376 their compatibility with a direct reverse transcription enzymatic step. This initial screening indicated  
377 that the addition of each of these detergents, alone, did not improve the assay compared to a direct  
378 assay performed on a crude commercial VTM sample. By contrast, the addition of PK improved the  
379 detection with all the detergents tested, in agreement with a recent study (28). Of note, a  
380 combination of Triton X-100 with Brij™ O10, in the absence of PK, had a similar effect, by decreasing  
381 the Cqs for the 3 SARS-CoV-2 primers/probes. (Fig. 4A) Brij™ O10 contains Oleth-10, a  
382 polyoxyethylene oleyl ether used in aqueous emulsions that may increase solubilization of the viral  
383 capsid or contribute to stabilization of the emulsion. When we tested the three best direct protocols  
384 on a cohort of samples collected in saline solution, we could not confirm the detection improvement  
385 observed in the screening assay using both lysis buffers (Tween-20 / PK and Triton X-100 / Brij™ O10,  
386 Fig. 5). This discrepancy may arise from the fact that the screened samples were collected in a  
387 commercial VTM while the cohort experiment was performed on biopsies collected in saline buffer.  
388 This difference suggests a complex interaction between the various constituents of this VTM (pH,  
389 nature of the medium, presence of albumin, gelatin, anti-bacterial agents, for examples), and the  
390 different detergents and emulsifiers tested. Further developments are definitely required to fine  
391 tune specific combinations of detergents and emulsifiers with specific VTMs. In any case, our data  
392 clearly indicate that a direct qPCR method using saline as a VTM and a basic TE buffer followed by a 5  
393 min inactivation step at 95°C shows almost identical performance to that of a classical extraction-

394 based assay, allowing notably the identification of samples with high Cq values (Fig. 5). Considering  
395 that SARS-CoV-2 remains detectable in phosphate buffer for up to a month when stored at various  
396 temperatures (29), our data supports the use of PBS, as an alternative to VTM for direct SARS-CoV-2  
397 testing. We further confirm and refine previous studies showing that testing for SARS-CoV-2 infection  
398 can be performed with simplified protocols omitting RNA extraction steps without major loss in  
399 accuracy (8, 9). Based on the data presented here, the simplest sample collection and preparation for  
400 direct RT-qPCR COVID-19 test would be to sample the swab into a small volume (around 0.5 mL) of  
401 TE buffer at a pH of 7.0 to limit dilution of the virus, supplemented with yeast tRNA or RNase  
402 inhibitors to increase RNA stability. This type of buffer was recently shown to have an excellent  
403 capacity to preserve the SARS-CoV-2 signal (9, 29). Lysis buffers containing detergents would provide  
404 the possibility to fully inactivate the virus, allowing a rapid and safe handling of the clinical samples,  
405 but our data indicate that their efficiency may vary depending on clinical sample processing and  
406 should be further optimized.

407 We propose that such a direct qPCR procedure may be especially useful for massively scaling up  
408 SARS-CoV-2 testing. We believe that this protocol can be very efficiently coupled with pooling  
409 approaches (30), to screen asymptomatic individuals in communities at risk. It would provide an  
410 additional tool to enhance testing capacity and affordability across the world, as widely  
411 recommended by the Health Community worldwide, such as the “all-in” approach to testing recently  
412 proposed (4).

413 **Acknowledgments:** Supported by funds from the “Centre National de la Recherche Scientifique”  
414 (CNRS), the “Université Côte d’Azur”, the French “French Defence Innovation Agency – Agence de  
415 l’Innovation de Défense “ (project “Safe and direct COV-2 qPCR Test”) and the Département des  
416 Alpes Maritimes (COVID-19 Health program). JF is supported by the Cancéropole PACA and CL is  
417 supported by Plan Cancer 2018 « ARN non-codants en cancérologie: du fondamental au  
418 translationnel » (number 18CN045). The Biomark equipment was funded by Canceropole PACA and

419 France Génomique (Commissariat aux Grands Investissements: ANR-10-INBS-6 09–03, ANR-10-INBS-  
420 09–02). We thank our colleagues from the IPMC and the Délégation CNRS Côte d’Azur for their help  
421 and support, especially Catherine Lecalvez, Michel Bordes, Nadine Pagliano and Simon Szmidt for  
422 administrative support and gratefully acknowledge the staff from the Département de Pneumologie  
423 of the Nice Hospital, especially Jennifer Griffonet and Charlotte Maniel. We also thank the technical  
424 support of the UCA GenomiX platform and MICA imaging facility of the University Côte d’Azur. We  
425 are grateful to Bayer SAS for loan of equipment and reagents as well as to Dr Pol-Henri Guivarch and  
426 Alexandre Romain (Agence Régionale De Santé PACA) for their support and interesting discussion.  
427 We are also grateful to the “Propagate” consortium, notably Syril D. Pettit, Emily A. Bruce, Jason W  
428 Botten and Keith R. Jerome for their support and fruitful discussion.

429

#### 430 **Author Contribution**

431 Conceived and designed experiments: **JF, CL, PB, LEZ, BM**. Performed the experiments: **JF, CL, LEZ**.  
432 Analyzed the data: **JF, CL, LEZ, BM**. Contributed reagents/materials/sample: **PB, SL, LN, SH, AD, MR,**  
433 **PT, ALC, CHM, DR, JLH, JLN**. Wrote paper: **JF, CL, GV, LEZ, BM**

434

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- 536
- 537

538 **Table 1. List of primers/probe for virus detection.** TaqMan probes are labeled at the 5'-end with the  
 539 reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) at the 3'-  
 540 end.  
 541  
 542  
 543

Country	Target	Name	Name used in this study	Sequence 5' to 3'
USA CDC,	N Gene	2019-nCoV_N1-F	N1	GACCCCAAATCAGCGAAAT
		2019-nCoV_N1-R		TCTGGTTACTGCCAGTTGAATCTG
		2019-nCoV_N1-P		ACCCCGCATTACGTTTGGTGGACC
	N Gene	2019-nCoV_N2-F	N2	TTACAAACATTGGCCGCAAA
		2019-nCoV_N2-R		GCGCGACATTCCGAAGAA
		2019-nCoV_N2-P		ACAATTTGCCCCAGCGCTTCAG
	N Gene	2019-nCoV_N3-F	N3	ATCACATTGGCACCCGCAATCCTG
		2019-nCoV_N3-R		AGATTTGGACCTGCGAGCG
		2019-nCoV_N3-P		TTCTGACCTGAAGGCTCTGCGCG
Charité, Germany	E gene	E_Sarbeco_F1	E	ACAGGTACGTTAATAGTTAATAGCGT
		E_Sarbeco_R2		ATATTGCAGCAGTACGCACACA
		E_Sarbeco_P1		ACACTAGCCATCCTTACTGCGCTTC G
China CDC,	Orf1 / Rdrp gene	ORF1ab-F	ORF1a b	CCCTGTGGGTTTTACTTAA
		ORF1ab-R		ACGATTGTGCATCAGCTGA
		ORF1ab-P		CCGTCTGCGGTATGTGAAAGGTTA TGG
Japan	N Gene	NIID_2019- nCoV_N_F2	N	AAATTTGGGGACCAGGAAC
		NIID_2019- nCoV_N_R2		TGGCAGCTGTGTAGGTCAAC
		NIID_2019- nCoV_N_P2		ATGTCGCGCATTGGCATGGA

544

545 **Table 2. List of primers/probe for cellular genes detection.** TaqMan probes are labeled at the 5'-end with  
 546 the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) at the 3'-  
 547 end.

548  
 549

Target	Name	Name used in this study	Sequence 5' to 3'
RnaseP	CDC-RP-F	RNP	AGATTTGGACCTGCGAGCG
	CDC-RP-R		GAGCGGCTGTCTCCACAAGT
	CDC-RP-P		TTCTGACCTGAAGGCTCTGCGCG
TMPRSS2	TMPRSS2 Forward	TMPRSS2	TATAGCCTGCGGGGTCAAC
	TMPRSS2 Reverse		CACTCGGGGGTGATGATGG
	TMPRSS2 Probe		TCAAGCCGCCAGAGCAGGATCGT
ACE2	ACE2 Forward	ACE2	GGCTCCTTCTCAGCCTTGTT
	ACE2 Reverse		GGTCTTCGGCTTCGTGGTTA
	ACE2 Probe		TGCTGCTCAGTCCACCATTGAGG
IL1a	IL1-a Forward	IL1a	CATTGGCGTTTGAGTCAGCA
	IL1-a Revrse		CATGGAGTGGGCCATAGCTT
	IL1-a Probe		GTCAAGATGGCCAAAGTTCAGACA
IL1b	IL1b Forward	IL1b	CAGAAGTACCTGAGCTCGCC
	IL1b Reverse		AGATTCGTAGCTGGATGCCG
	IL1b Probe		CCAGGACCTGGACCTCTGCC
CXCL8	CXCL8 Forward	CXCL8	TGGACCCCAAGGAAAAGTGG
	CXCL8 Reverse		TTTGCTTGAAGTTTCACTGGCA
	CXCL8 Probe		GTGCAGAGGGTTGTGGAGAAGTTT
IL6	IL6 Forward	IL6	TGCAATAACCACCCCTGACC
	IL6 Reverse		GTGCCATGCTACATTTGCC
	IL6 Probe		TGCCAGCCTGCTGACGAAGC
IFNb1	IFNb1 Forward	IFNb1	AGTAGGCGACACTGTTCTGTG
	IFNb1 Reverse		GCCTCCCATTCAATTGCCAC
	IFNb1 Probe		TGCTCTCCTGTTGTGCTTCTCCA
IFIT1	IFIT1 Forward	IFIT1	GATCTCAGAGGAGCCTGGCTAA
	IFIT1 Reverse		TGATCATCACCATTTGACTCATGG
	IFIT1 Probe		CAAAACCCTGCAGAACGGCTGCC

550  
 551



552 **Legends to Figures**

553

554 **Figure 1. Performance and validation of the Biomark-HD SARS-CoV-2 qPCR assay.**

555 **A.** Titration of the diluted (N) nucleocapside spike-in transcript. A serial dilution of the synthetic N  
556 transcript from  $2 \cdot 10^4$  to 2 copy was performed and processed through the Biomark-HD protocol.  
557 Correlation and amplification curves of detected Cq values according to synthetic N transcript copy  
558 number obtained with N1 primers/probe are shown. **B.** Amplification curves showing the range of  
559 detection of a SARS-Cov2 positive clinical sample serial dilution with N1 and E primers/probe. **C.**  
560 Typical amplification curves showing the combination of two primers/probe sets on 3 SARS-Cov2  
561 positive clinical samples. Mix #1: E, N1, human RNP. Mix #2: E, N, ORF1ab and human RNP. **C.**  
562 Validation of the Biomark-HD protocol on a cohort of 18 biopsies from positive patients. The  
563 correlation of the Cq values obtained for the N, E and the ORF1ab genes are presented. The data  
564 presented are representative of at least two independent experiments performed in quadruplicate.

565

566 **Figure 2. Optimization of the assay.**

567 **A.** Total RNA from 18 clinical samples with a wide range of SARS-CoV-2 infection were subjected to  
568 either a Two-step reaction (red circles; consecutive Reverse Transcription and Pre-amplification) or  
569 One-step reaction (blue circles; combined Reverse Transcription and Pre-amplification). Quantitative  
570 PCR reactions were performed on the Biomark-HD using cellular (RNP) and viral (N, E, ORF1ab)  
571 primers/probe sets. **B.** Total RNA from 18 clinical samples with a wide range of SARS-CoV-2 infection  
572 were extracted with either the miRNeasy Advanced Serum Plasma Kit (red circles) or the Virus  
573 QIAamp Viral RNA kit (blue circles). RNAs were processed using the One step reaction. Quantitative  
574 PCR reactions were performed on the Biomark-HD using cellular (RNP) and viral (N, E, ORF1ab)  
575 primers/probe sets. The Cq presented are representative of two independent experiments  
576 performed in quadruplicate.

577

578 **Figure 3. Use of the Biomark-based protocol to analyze the host Response to SARS-CoV-2 infection**  
579 **at the mRNA and microRNA levels**

580 **A.** Overview of the analysis strategy on 72 patient samples. They were divided into 4 groups,  
581 according to their viral load, from negative, weak (Cq for viral probes >20), medium (20 > Cq for viral  
582 probes >10) and strong (Cq for viral probes < 10) SARS-CoV-2 positive. **B.** Typical amplification curves  
583 of the different genes (cellular, viral and micro-RNA) on three different SARS-Cov2 patient status. **C.**  
584 Modulation of cellular markers in the different groups of patients according to their SARS-CoV-2 viral  
585 load. IFIT1 expression was statistically elevated ( $p < 0.001$ ) in the strong COVID-19-positive samples  
586 compared to the three other groups considered separately. The Cq presented are representative of  
587 two independent experiments performed in quadruplicate.

588

589 **Figure 4. Detection of viral and cellular genes using inactivating lysis buffers-based protocols.**

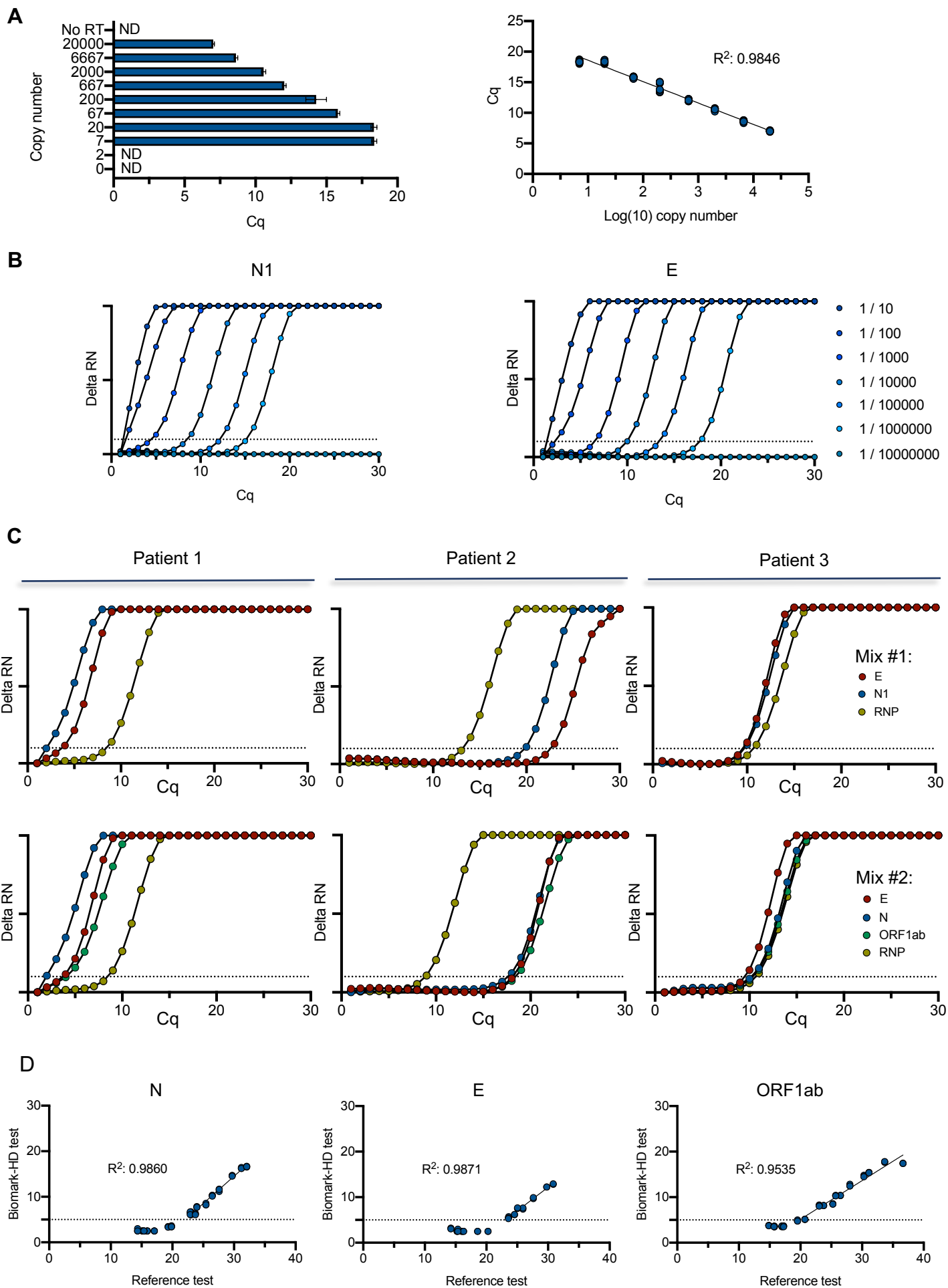
590 Two clinical samples of known COVID-19 status (one positive and one negative) sampled in a  
591 commercial VTM medium, were aliquoted and then treated in parallel using the indicated detergent  
592 conditions (selected from Fig. S5) in the presence or absence of PK. RNA was extracted as a control.  
593 Biomark-HD RT-qPCR was performed using **(A)** viral (N, E, ORF1ab) and **(B)** cellular (ACE2, IFIT1, IL6,  
594 TMPRSS and RNP) primers/probe sets. Legend: +: 10 min at 65°C; ++: 10 min at 65°C and 5 min at 95°C;  
595 TX: Triton X-100; QE: Quick Extract™ DNA Extraction Solution. The Cq presented are representative  
596 of two independent experiments performed in quadruplicate.

597

598 **Figure 5. Correlation of the detection of two viral genes (N, ORF1ab) obtained using a commercial**  
599 **COVID-19 detection kit and extraction-based or direct Biomark-HD assays.** We used a set of 17

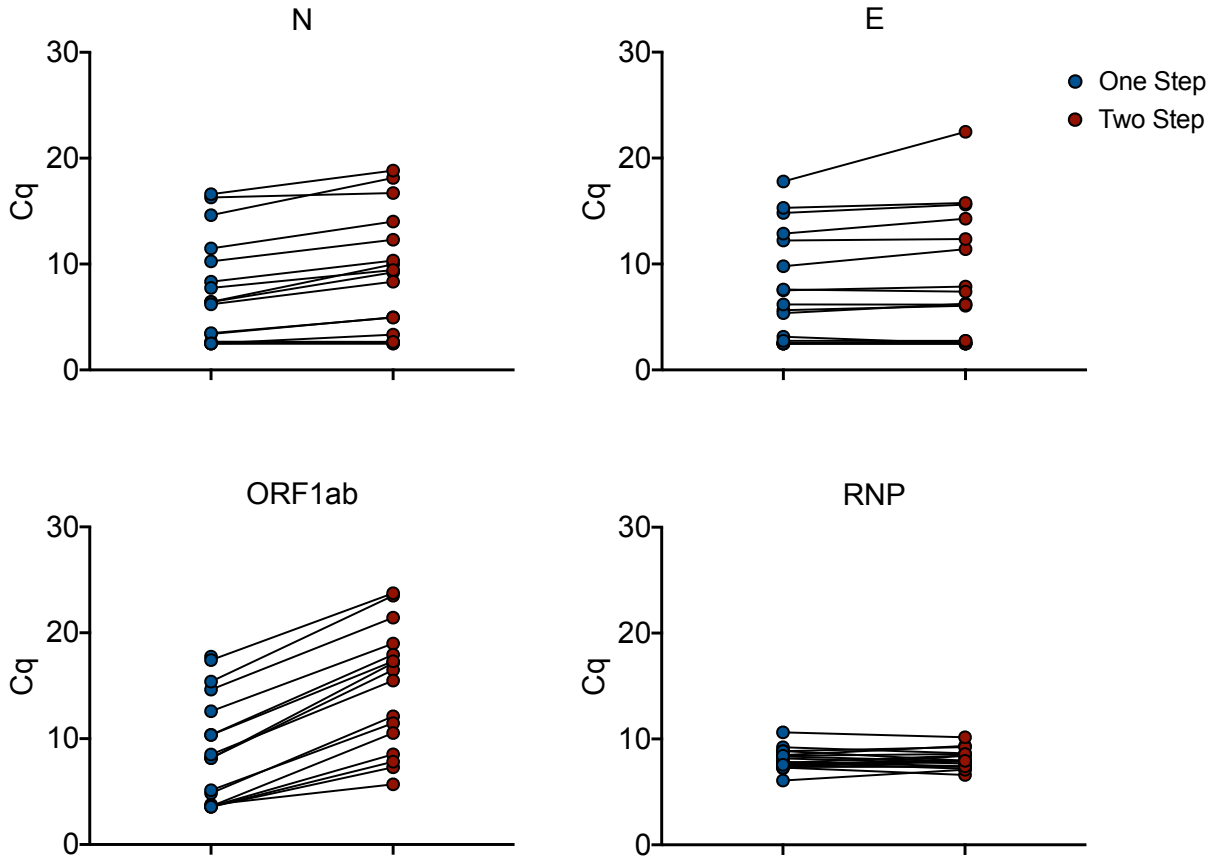
600 clinical samples from SARS-CoV-2-diagnosed patients collected in saline solution with a wide range of  
601 Cq values. To control pH conditions and limit RNA degradation, all samples were diluted 2X in TE  
602 buffer. RNA was extracted (as a control) or samples were treated using Tween 20 and PK (Tw + PK) or  
603 Triton X-100 and Brij010 detergent solutions (Tx + Brij010). The different RT-qPCR Biomark methods

604 were compared with the GeneFirst™ COVID-19 method as a reference. The correlation between the  
605 Cq obtained with the two methods is presented.

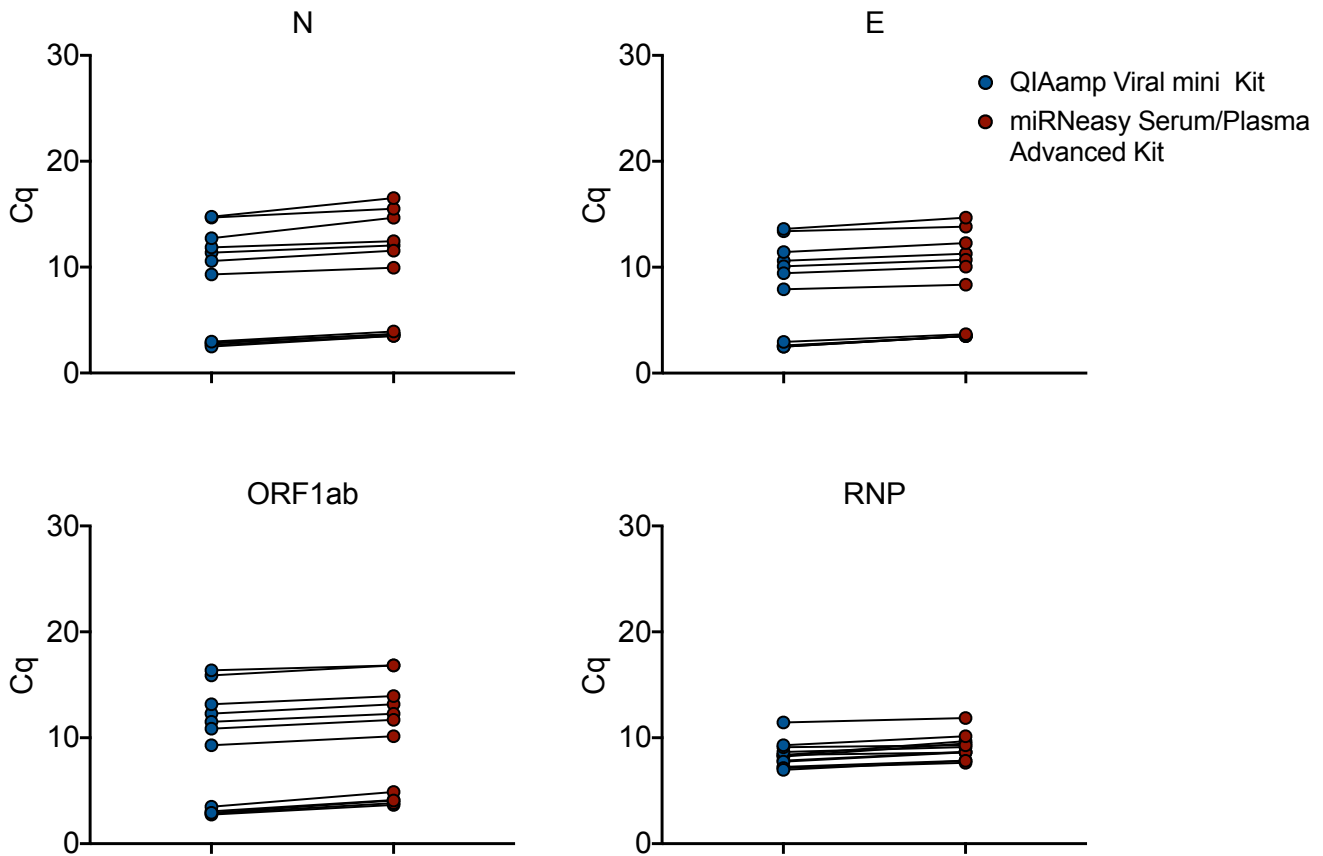


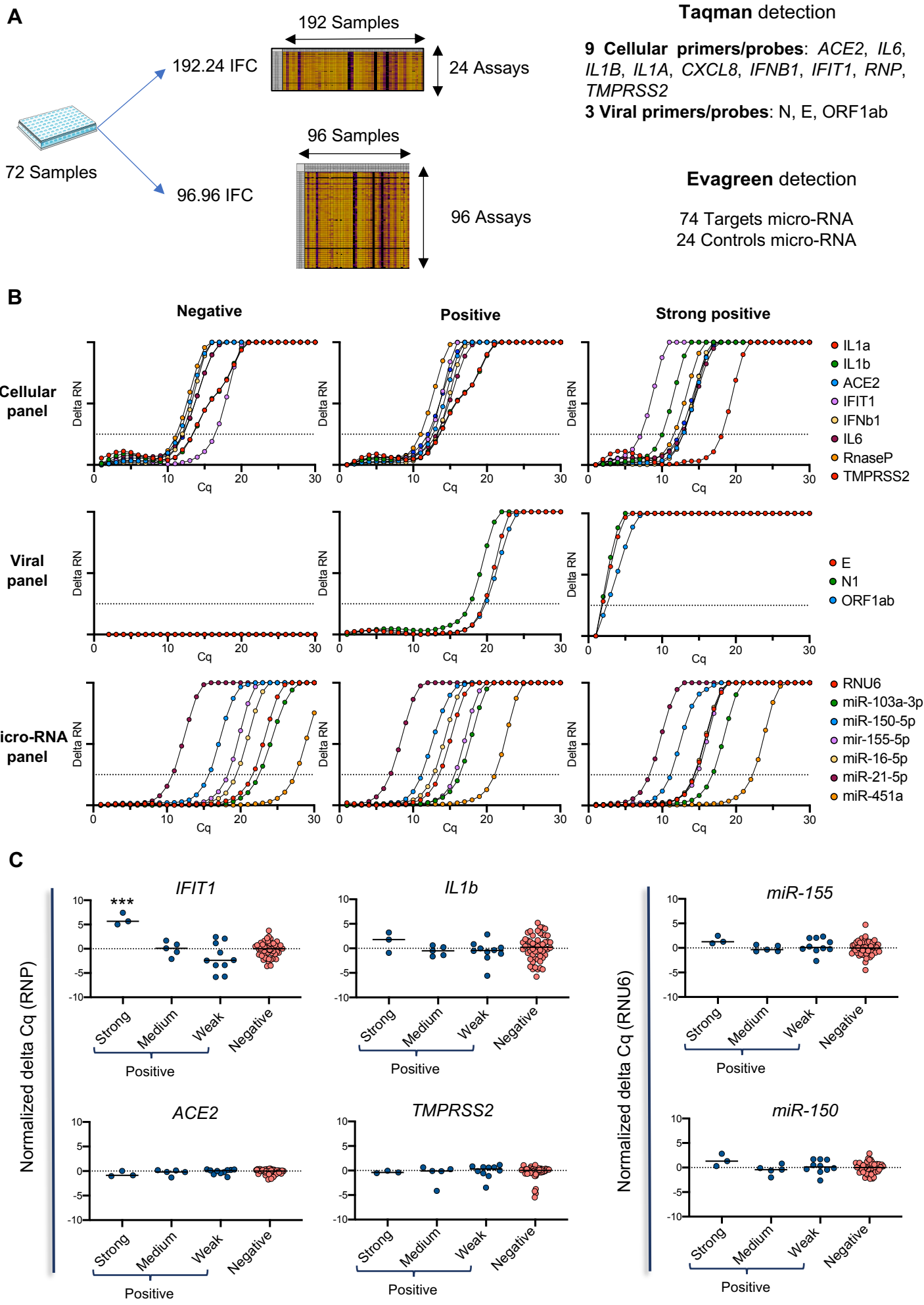
# Fassy, Lacoux et al Fig. 2

**A**

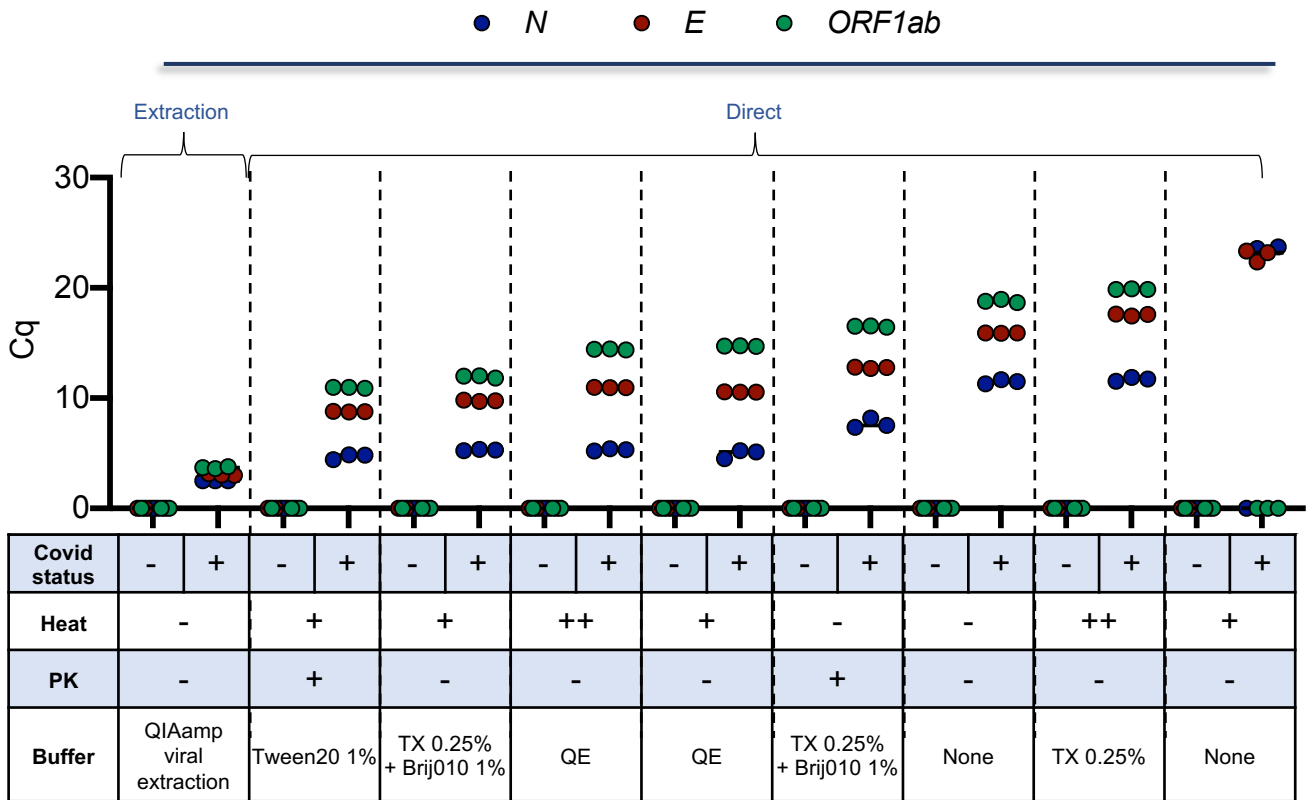


**B**

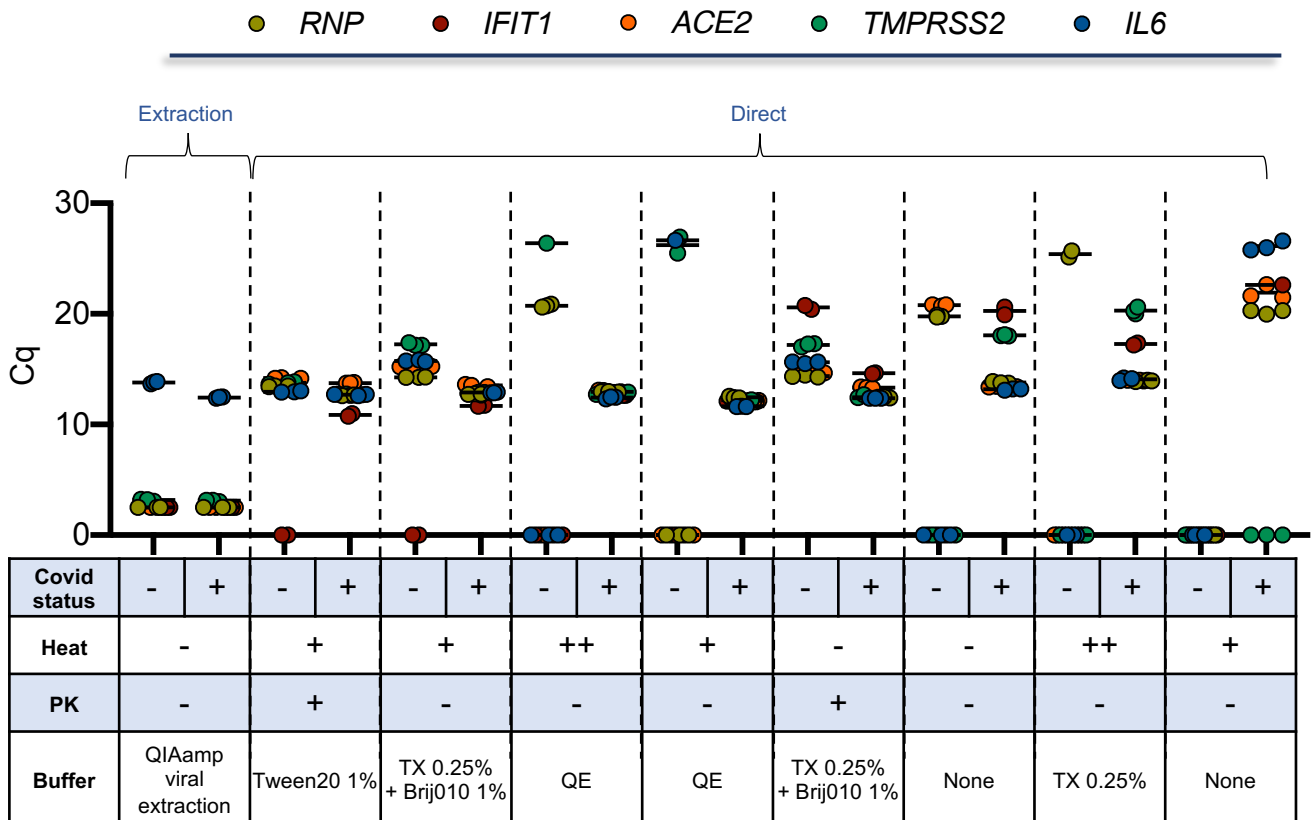




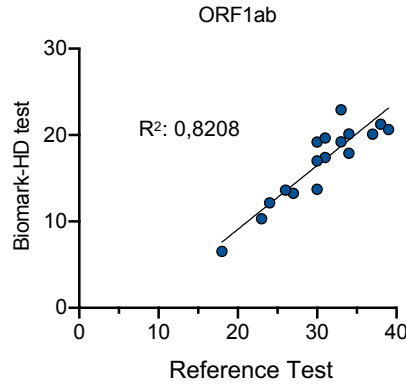
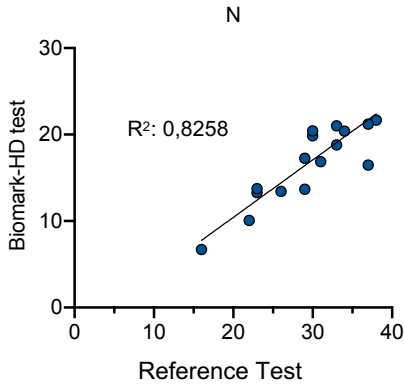
A



B



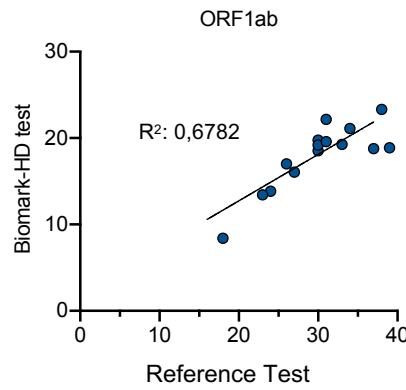
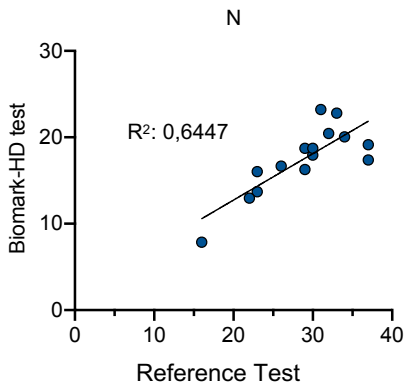
Extraction



Detection sensitivity

DP	16
N	0
ORF1ab	1
Total	17/17

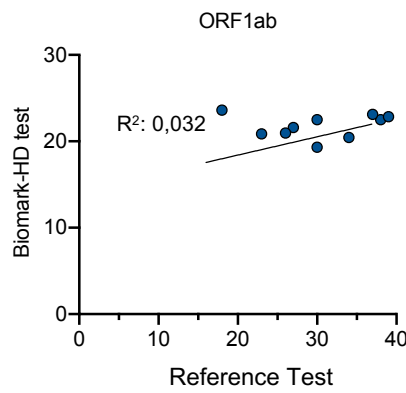
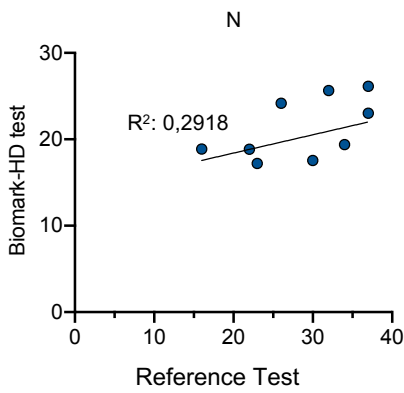
TE



Detection sensitivity

DP	14
N	1
ORF1ab	1
Total	16/17

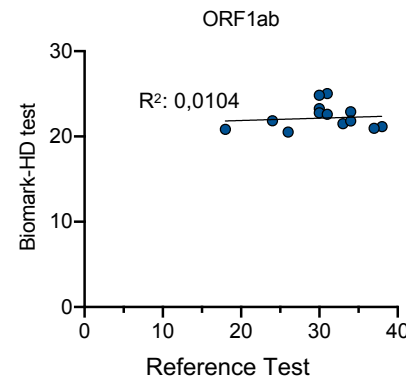
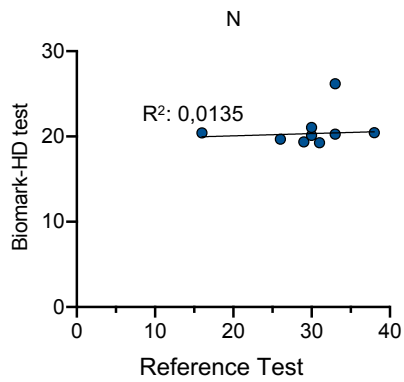
Tw + PK



Detection sensitivity

DP	8
N	1
ORF1ab	2
Total	11/17

Tx + Brij010



Detection sensitivity

DP	8
N	1
ORF1ab	5
Total	14/17