

1 **SARS-CoV-2 RNA quantification using droplet digital RT-PCR**

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44 **Abstract**

45
46 Quantitative viral load assays have transformed our understanding of – and ability to
47 manage – viral diseases. They hold similar potential to advance COVID-19 control and
48 prevention, but SARS-CoV-2 viral load tests are not yet widely available. SARS-CoV-2
49 molecular diagnostic tests, which typically employ real-time reverse transcriptase-polymerase
50 chain reaction (RT-PCR), yield semi-quantitative results only. Reverse transcriptase droplet
51 digital PCR (RT-ddPCR), a technology that partitions each reaction into 20,000 nanolitre-sized
52 droplets prior to amplification, offers an attractive platform for SARS-CoV-2 RNA
53 quantification. We evaluated eight primer/probe sets originally developed for real-time RT-PCR-
54 based SARS-CoV-2 diagnostic tests for use in RT-ddPCR, and identified three (Charité-Berlin
55 E-Sarbeco and Pasteur Institute IP2 and IP4) as the most efficient, precise and sensitive for RT-
56 ddPCR-based SARS-CoV-2 RNA quantification. Analytical efficiency of the E-Sarbeco
57 primer/probe set, for example, was ~83%, while assay precision, as measured by the coefficient
58 of variation, was ~2% at 1000 input copies/reaction. Lower limits of quantification and detection
59 for this primer/probe set were 18.6 and 4.4 input SARS-CoV-2 RNA copies/reaction,
60 respectively. SARS-CoV-2 RNA viral loads in a convenience panel of 48 COVID-19-positive
61 diagnostic specimens spanned a $6.2\log_{10}$ range, confirming substantial viral load variation *in*
62 *vivo*. We further calibrated RT-ddPCR-derived SARS-CoV-2 E gene copy numbers against cycle
63 threshold (C_t) values from a commercial real-time RT-PCR diagnostic platform. The resulting
64 log-linear relationship can be used to mathematically derive SARS-CoV-2 RNA copy numbers
65 from C_t values, allowing the wealth of available diagnostic test data to be harnessed to address
66 foundational questions in SARS-CoV-2 biology.

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68

69 **Introduction**

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71 Quantitative viral load assays have revolutionized our ability to manage viral diseases (1-

72 6). While not yet widely available for SARS-CoV-2, quantitative assays could advance our

73 understanding of COVID-19 biology and inform infection prevention and control measures (7,

74 8). Most SARS-CoV-2 molecular diagnostic assays however, which use real-time reverse

75 transcriptase PCR (RT-PCR) to detect one or more SARS-CoV-2 genomic targets using

76 sequence-specific primers coupled with a fluorescent probe, are only semi-quantitative. These

77 tests produce cycle threshold (C_t) values as readouts, which represent the PCR cycle where the

78 sample began to produce fluorescent signal above background. While each C_t value decrement

79 corresponds to a roughly two-fold higher viral load (due to the exponential nature of PCR

80 amplification), C_t values cannot be directly interpreted as SARS-CoV-2 viral loads without

81 calibration to a quantitative standard (9). Rather, C_t values are interpreted as positive,

82 indeterminate or negative based on assay-specific cutoffs and evolving clinical guidelines. Due

83 to differences in nucleic acid extraction method, viral target and other parameters, C_t values are

84 also not directly comparable across assays or technology platforms.

85 Reverse transcriptase droplet digital PCR (RT-ddPCR) offers an attractive platform for

86 SARS-CoV-2 RNA quantification (10, 11). Like real-time RT-PCR, ddPCR employs target-

87 specific primers coupled with a fluorescent probe, making it relatively straightforward to adapt

88 assays. In ddPCR however, each reaction is fractionated into 20,000 nanolitre-sized droplets

89 prior to massively parallel PCR amplification. At end-point, each droplet is categorized as

90 positive (target present) or negative (target absent), allowing for absolute target quantification

91 using Poisson statistics. This sensitive and versatile technology has been used for mutation

92 detection and copy number determination in the human genome (12), target verification

93 following genome editing (13), and copy number quantification for viral pathogens (14-19).
94 Several real-time RT-PCR SARS-CoV-2-specific primer/probe sets have been used in RT-
95 ddPCR (10, 11, 20-22) with results achieving high sensitivity in some reports (11, 21, 23-25), but
96 few studies have rigorously evaluated SARS-CoV-2-specific primer/probe set performance in
97 RT-ddPCR using RNA as a template. Furthermore, no studies to our knowledge have calibrated
98 SARS-CoV-2 viral loads to diagnostic test C_t values. Here, we evaluate eight SARS-CoV-2-
99 specific primer/probe sets originally developed for real-time RT-PCR (26), for use in RT-
100 ddPCR. We also derive a linear equation relating RT-ddPCR-derived SARS-CoV-2 viral loads
101 and real-time RT-PCR-derived C_t values for a commercial diagnostic assay, the LightMix®
102 Modular SARS-CoV (COVID19) E-gene assay, allowing conversion of existing COVID-19
103 diagnostic results to viral loads.

104 **Materials and Methods**

105

106 **Primer and Probe Sets**

107 Eight SARS-CoV-2-specific primer/probe sets developed for real-time RT-PCR COVID-
108 19 diagnostic assays (26) were assessed for use in RT-ddPCR (Table 1). These included the
109 Charité-Berlin E gene ('E-Sarbeco') set (27), the Pasteur Institute RdRp IP2 and IP4 sets ('IP2'
110 and 'IP4', respectively) (28), the Chinese Centre for Disease Control ORF and N gene sets
111 ('China-ORF' and 'China-N', respectively) (29), the Hong Kong University ORF and N gene
112 sets ('HKU-ORF' and 'HKU-N', respectively) (30), and the US-CDC-N1 set (31).

113 **SARS-CoV-2 Synthetic RNA standards**

114 RT-ddPCR assays were evaluated using commercial synthetic SARS-CoV-2 RNA
115 standards comprising six non-overlapping 5,000 base fragments of equal quantities encoding the
116 Wuhan-Hu-1 SARS-CoV-2 genome (Control 2, Genbank ID MN908947.3; Twist Biosciences,
117 supplied at approximately 1 million copies/fragment/ μ l). To avoid degradation, RNA standards
118 were stored at -80°C and thawed only once, immediately before use, to perform the analytical
119 efficiency, precision, analytical sensitivity and dynamic range analyses described herein.
120 Moreover, to mimic nucleic acid composition of a real biological specimen, all assays employing
121 these standards were supplemented with a consistent, physiologically relevant amount of nucleic
122 acid extracted from pooled remnant SARS-CoV-2-negative nasopharyngeal swabs
123 (Supplementary Figure 1). Briefly, pooled viral transport medium was extracted in 1ml aliquots
124 on the BioMerieux NucliSens® EasyMag®, eluted in 60 μ l and re-pooled. The resulting material
125 contained DNA from on average 2,200 human cells/ μ l (as quantified using human RPP30 DNA
126 copy numbers by ddPCR as described in (32)) and 4,400 human RNase P copies/ μ l extract (as

127 quantified by RT-ddPCR as described in (33)), concentrations that are in line with human DNA
128 and RNA levels recovered on nasopharyngeal swabs (32, 33).

129 **Reverse transcriptase droplet digital PCR (RT-ddPCR) for SARS-CoV-2 quantification**

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131 RT-ddPCR reactions were performed by combining relevant SARS-CoV-2 RNA
132 template with target-specific primers and probe (900nM and 250nM, respectively, Integrated
133 DNA Technologies; Table 1), One-Step RT-ddPCR Advanced Kit for Probes Supermix, Reverse
134 Transcriptase and DTT (300nM) (all from BioRad), XhoI restriction enzyme (New England
135 Biolabs), background nucleic acid (for reactions employing synthetic RNA template only, see
136 above) and nuclease free water. Droplets were generated using an Automated Droplet Generator
137 (BioRad) and cycled under primer/probe set-specific conditions (see below and Figure 1).
138 Analysis was performed on a QX200 Droplet Reader (BioRad) using QuantaSoft software
139 (BioRad, version 1.7.4).

140 **Thermal cycling temperature optimization**

141 For each primer/probe set, acceptable thermal cycling temperature ranges for reverse
142 transcription (RT) and PCR annealing/extension were determined by modifying the
143 manufacturer-recommended default conditions, which are 42-50°C for 1 hour (for reverse
144 transcription); 95°C for 10 minutes; 40 cycles of (94°C for 30 seconds followed by 50-63°C for
145 1 minute); 98°C for 10 minutes and 4°C infinite hold. To determine acceptable temperature
146 ranges for reverse transcription, a thermal gradient from 42-51.5°C was performed while fixing
147 the annealing/extension step at 52°C. Using the optimized reverse transcription temperature, a
148 thermal gradient from 50-63°C was then performed to identify acceptable annealing/extension
149 temperature ranges. Temperatures that produced insufficient separation of positive from negative
150 droplets or non-specific amplification were deemed unacceptable, as were those that produced

151 consecutive 95% confidence intervals of copy number estimates outside those of the maximal
152 point-estimate.

153 **Analytical Efficiency and Precision**

154 The analytical efficiency of each primer/probe set to quantify SARS-CoV-2 RNA by RT-
155 ddPCR was determined using synthetic SARS-CoV-2 RNA standards at 1000 and 100 input
156 copies. A minimum of three (maximum four) technical replicates were performed at each
157 concentration. Analytical efficiency was calculated by dividing the measured SARS-CoV-2 copy
158 number by the expected input copy number, and multiplying by 100. Precision was expressed as
159 the coefficient of variation (CV), expressed as a percentage, across technical replicates.

160 **Linear Dynamic Range**

161 The linear dynamic range (LDR) of each primer/probe set of interest was determined
162 across a serial 1:2 dilution series from 114,286 to 1.2 SARS-CoV-2 RNA copies/reaction. This
163 range of concentrations was chosen as it crosses the entire range of recommended input copies
164 for a ddPCR reaction seeking to quantify the target of interest (34). Reactions were performed in
165 duplicate. The upper and lower limits of quantification of (ULOQ and LLOQ, respectively) were
166 defined as the upper and lower boundaries of the concentration range over which the relationship
167 between measured and input SARS-CoV-2 RNA copies was linear. This was determined by
168 iteratively restricting the range of concentrations included in the linear regression of measured
169 versus input SARS-CoV-2 RNA copies to identify that which maximized the coefficient of
170 determination (R^2) value and minimized the residuals.

171 **Assay Analytical Sensitivity**

172 Assay analytical sensitivity, defined as the Lower Limit of Detection (LLOD), was
173 determined for primer/probe sets of interest by serially diluting synthetic SARS-CoV-2 RNA

174 standards to between 47.6 and 0.74 SARS-CoV-2 RNA copies/reaction. Between 6 and 18
175 technical replicates were performed for each dilution and results were analyzed using probit
176 regression. The LLOD, determined through interpolation of the probit curve, was defined as the
177 concentration of input SARS-CoV-2 RNA in a reaction where the probability of detection was
178 95%.

179 **SARS-CoV-2 RNA quantification in biological specimens, and relationship to C_t value**

180 Optimized RT-ddPCR assays were applied to a convenience sample of 48 consecutive
181 remnant SARS-CoV-2-positive diagnostic nasopharyngeal swab specimens that were originally
182 submitted to the St. Paul's Hospital Virology Laboratory in Vancouver, Canada for diagnostic
183 testing using the Roche cobas® SARS-CoV-2 assay. For these samples, total nucleic acids were
184 re-extracted from 250µl remnant media using the BioMerieux NucliSens® EasyMag® and
185 eluted in 50µl. Eluates were aliquoted and frozen at -80°C prior to single use. SARS-CoV-2 copy
186 numbers were quantified by RT-ddPCR as described above. As our main goal was to
187 characterize the relationship between C_t values and SARS-CoV-2 RNA levels without
188 confounding by extraction platform, quantity of input material or SARS-CoV-2 genomic target,
189 we re-tested these extracts using a commercial real-time RT-PCR SARS-CoV-2 diagnostic assay
190 that uses the E-Sarbeco primer/probe set (27): the LightMix® 2019-nCoV real-time RT-PCR
191 assay E-gene target (Tib-Molbiol), implemented on LightCycler 480 (Roche Diagnostics).
192 Finally, to be responsive to a recent recommendation that SARS-CoV-2 viral loads be reported
193 in terms of SARS-CoV-2 RNA copies per human cell equivalents (9), we measured human
194 cells/µl extract by ddPCR as previously described (32) and additionally reported results as
195 SARS-CoV-2 RNA copies/1,000 human cells.

196 **Statistical Analysis**

197 Statistical analysis was performed using GraphPad Prism (Version 8) or Microsoft Excel
198 (Version 14.7.2).

199 **Ethical Approval**

200 This study was approved by the Providence Health Care/University of British Columbia
201 and Simon Fraser University Research Ethics Boards under protocol H20-01055.

202

203 **Results**

204 **Thermal cycling optimization for SARS-CoV-2 quantification by RT-ddPCR**

205 Eight primer/probe sets originally developed for SARS-CoV-2 diagnostic testing by real
206 time RT-PCR were evaluated for use in RT-ddPCR (Table 1). As these primer/probe sets vary in
207 sequence, amplicon length and SARS-CoV-2 genomic target, we first determined the acceptable
208 temperature ranges for reverse transcription (RT) and PCR annealing/extension. Most
209 primer/probe sets were tolerant to a wide temperature range, and background signal was
210 essentially zero at all temperatures tested (Figure 1). The E-Sarbeco primer/probe set for
211 example produced consistent amplitude profiles, copy number estimates and essentially zero
212 background at annealing/extension temperatures ranging from 50-63°C (Figure 1A and data not
213 shown). The HKU-ORF primer/probe performed acceptably over a 50-60.5°C
214 annealing/extension range, but positive and negative droplet separation was insufficient at higher
215 temperatures (Figure 1B). Acceptable temperature ranges for each primer/probe set are shown in
216 Figure 1C. All subsequent experiments were performed at RT 42.7°C and annealing/extension
217 50.9°C except those for HKU-ORF and US-CDC-N1, which were performed at RT 45.7°C and
218 annealing/extension 55.1°C as informed by initial qualitative assessments.

219 **Analytical Efficiency and Precision of SARS-CoV-2 quantification by RT-ddPCR**

220 We next evaluated the analytical efficiency of SARS-CoV-2 RNA quantification for each
221 primer/probe set, calculated as the percentage of input viral RNA copies detected by the assay.
222 We also evaluated precision, calculated as the dispersion of measured copies around the mean
223 (coefficient of variation, CV). Analytical efficiency and precision were evaluated at 1000 and
224 100 SARS-CoV-2 RNA target input copies. At 1000 input copies, primer/probe set analytical
225 efficiency ranged from 83% (E-Sarbeco) to 15% (US-CDC-N1) (Figure 2A). At 100 copies, the

226 analytical efficiency hierarchy was identical, with values ranging from 74% (E Sarbeco) to 12%
227 (US-CDC-N1). Of all primer/probe sets evaluated, the E-Sarbeco, IP2 and IP4 sets had the
228 highest analytical efficiencies by a substantial margin. At 1000 and 100 target copies, E-Sarbeco
229 analytical efficiency was 83% (95% Total Poisson Confidence Interval [CI]: 79- 87%) and 74%
230 (95% CI: 63- 84%), respectively; IP2, analytical efficiency was 70% (95% CI: 67- 73%) and
231 55% (95% CI: 46- 64%), respectively; and IP4 analytical efficiency was 69% (95% CI: 66- 72%)
232 and 59% (95% CI: 50-69%), respectively. In contrast, analytical efficiency of the China-ORF
233 primer/probe set was only 46% and 39% at 1000 and 100 input copies, respectively, and the
234 analytical efficiencies of the remaining sets were less than 30% regardless of input copy number.
235 Furthermore, while measurement precision generally decreased at the lower template
236 concentration (35), the E-Sarbeco, IP2 and IP4 primer/probe sets were nevertheless among the
237 most precise, with coefficients of variation (CV) of less than 5% at 1,000 input copies and less
238 than 15% at 100 input copies (Figure 2B). Combined analytical efficiency and precision data
239 confirmed E-Sarbeco, IP2 and IP4 as the best-performing primer/probe sets in RT-ddPCR
240 (Figures 2C and 2D), so these were moved forward for further characterization.

241 **Reduced analytical efficiency when IP2 and IP4 are duplexed in RT-ddPCR**

242 As IP2 and IP4 were originally designed for duplexing in real-time RT-PCR (28), we
243 evaluated them in duplex for RT-ddPCR. Duplexing however decreased analytical efficiency,
244 from 70% to 52% (at 1000 input copies) and 55% to 37% (at 100 input copies) for IP2, and from
245 69% to 49% (at 1000 input copies) and 59% to 38% (at 100 input copies) for IP4 (Supplemental
246 Figure 2A). Duplexing also decreased precision (Supplemental Figure 2B). For IP2, CV
247 increased from 5% to 11% when duplexing at 1000 input copies, and from 15% to 25% when
248 duplexing at 100 input copies. For IP4, CV increased from 4% to 7% (1000 input copies) and

249 from 14% to 21% (100 input copies) with duplexing. Duplexing of these reactions is therefore
250 not recommended in RT-ddPCR, and all IP2 and IP4 assays were performed as single reactions.

251 **Linear Dynamic Range and Limits of Quantification of SARS-CoV-2 RNA by RT-ddPCR**

252 Droplet digital PCR can achieve absolute target copy number quantification without a
253 standard curve. To investigate the linear dynamic range (LDR) of quantification of the E-
254 Sarbeco, IP2 and IP4 assays, we set up 18 two-fold serial dilutions of synthetic SARS-CoV-2
255 RNA beginning at 114,286 copies/reaction (this copy number is obtained when 120,000 copies
256 are added to a 21 μ l reaction, of which 20 μ l is used for droplet generation) and ending with 2.32
257 copies/reaction. This input copy number range crosses nearly the entire manufacturer-
258 recommended template input range for ddPCR reactions seeking to quantify the target of interest,
259 which is 1- 100,000 copies/reaction (36).

260 The LDR of each assay was determined by iteratively restricting the range of
261 concentrations included in the linear regression of measured versus input SARS-CoV-2 RNA
262 copies to identify the range that maximized the R^2 value and minimized the residuals. For E-
263 Sarbeco, the regression spanning 18.6-114,286 input SARS-CoV-2 RNA copies per reaction, an
264 approximately 6,100-fold concentration range, yielded an R^2 value of 0.9995 (Figure 3A, left).
265 Restricting the linear regression to this range also minimized the residuals of all included data
266 points to $\pm 0.065 \log_{10}$ copies/reaction (Figure 3A, right). The IP2 assay, while less efficient than
267 E-Sarbeco, had the same estimated LDR of 18.6-114,286 input copies/reaction (Figure 3B, left).
268 This produced an R^2 value of 0.9995 and residuals within $\pm 0.065 \log_{10}$ copies/reaction across the
269 LDR (Figure 3B, right). The LDR of IP4 was estimated as 37.2- 114,286 input copies/reaction,
270 an approximately 3,000-fold range, which yielded an $R^2 = 0.9975$ and produced residuals within
271 $\pm 0.11 \log_{10}$ copies/reaction across this range (Figure 3C). For all three assays, 114,286 input

272 copies/reaction should be considered a conservative estimate of the upper limit of quantification,
273 as saturation of the RT-ddPCR reaction or loss of linearity was still not achieved at this
274 concentration.

275 **Lower Limit of Detection of SARS-CoV-2 RNA by RT-ddPCR**

276 We next determined the lower limit of detection (LLOD) of the E-Sarbeco, IP2 and IP4
277 RT-ddPCR assays (Figure 4). Probit regression analysis applied to serial dilutions of synthetic
278 SARS-CoV-2 RNA standards revealed the E-Sarbeco RT-ddPCR assay to be the most
279 analytically sensitive of the three, which is consistent with it also having the highest analytical
280 efficiency. Specifically, the estimated LLOD of the E-Sarbeco assay was 4.4 (95% Confidence
281 Interval [CI]: 2.4-5.7) SARS-CoV-2 RNA copies/reaction (Figure 4A). The estimated LLOD of
282 the IP2 assay was 7.8 (95% CI: 4.4-10.3) SARS-CoV-2 RNA copies/reaction (Figure 4B), while
283 that of IP4 was 12.6 (95% CI: 6.9-16.5) SARS-CoV-2 RNA copies per reaction (Figure 4C).

284 **SARS-CoV-2 viral loads in biological samples**

285 SARS-CoV-2 viral loads were measured in 48 confirmed SARS-CoV-2 positive samples
286 using the E-Sarbeco, IP2 and IP4 primer/probe sets (note that samples with original diagnostic
287 test C_t values <19 required RNA extracts to be diluted up to 1:200 prior to quantification to
288 ensure that input copies measurements fell within each assay's LDR). The results revealed that
289 SARS-CoV-2 RNA in these biological samples varied over a 6.2 \log_{10} range (Figure 5A).
290 Average copy numbers measured using the E-Sarbeco assay (which targets the E gene) were
291 higher than those using the IP2 and IP4 assays (which target ORF1a and ORF1b, respectively)
292 (Figure 5A). This is consistent with assay analytical efficiency (Figure 2) and *in vivo* coronavirus
293 RNA expression patterns, where transcripts covering the 3' end of the genome are more
294 abundant than those covering the 5' end (37-40). Specifically, the median E-gene copy number

295 was 5.1 (IQR 3.9- 5.7) \log_{10} copies/ μ l extract compared to a median of 4.9 (IQR 3.9- 5.5)
296 \log_{10} copies/ μ l extract for the IP2 target, and a median of 4.9 (IQR 3.9- 5.6) \log_{10} copies/ μ l
297 extract for the IP4 target. SARS-CoV-2 E-gene, IP2 and IP4 copy numbers in biological samples
298 correlated strongly with one another (Spearman's $\rho > 0.99$; $p < 0.0001$ for all pairwise analyses;
299 Figure 5BCD). Consistent with comparable ORF1a and ORF1b RNA transcript levels *in vivo*
300 (37, 38, 40), IP2 and IP4 copy numbers were also highly concordant (Lin's concordance
301 correlation coefficient, $\rho_c = 0.9996$ [95% CI: 0.9993- 0.9998]) (Figure 5D). Based on a recent
302 recommendation (9), we also report our results in terms of SARS-CoV-2 RNA copies per human
303 cell equivalents: results for E-Sarbeco spanned an 7-fold range from 1.05 to 7.3 \log_{10} SARS-
304 CoV-2 RNA copies/1,000 human cells, with IP2 and IP4 \log_{10} copy numbers lower, as expected
305 (Supplemental Figure 3A). The Spearman's correlation between absolute and human cell-
306 normalized viral loads was strong ($\rho = 0.9717$; $p < 0.0001$; Supplementary Figure 3B), which is
307 consistent with the assumption that the amount of biological material collected by
308 nasopharyngeal swabs is relatively consistent.

309 **Inferring SARS-CoV-2 viral loads from diagnostic C_t values**

310 Finally, we characterized the relationship between C_t values produced by a commercial
311 COVID-19 diagnostic platform and SARS-CoV-2 RNA copy numbers. We selected the
312 LightMix® 2019-nCoV real-time RT-PCR assay, E-gene target (Tib-Molbiol), implemented on a
313 LightCycler 480 (Roche Diagnostics) because commercial diagnostic reagents comprising the E-
314 Sarbeco primer/probe set exist for this platform (27) and because it takes purified nucleic acids
315 as input, thereby allowing direct comparison of results from the same starting material (real-time
316 RT-PCR platforms that take biological material as input are suboptimal for such a comparison
317 because the onboard extraction introduces an additional variable). As the C_t values reported for

318 the LightMix® assay are based on a 9µl extract input volume, our primary analysis reported RT-
319 ddPCR results in terms of SARS-CoV-2 copies equivalent (*i.e.* SARS-CoV-2 copies in 9µl of
320 extract), to allow direct conversion of C_t values to absolute viral copy numbers.

321 Sample C_t values ranged from 11.34-31.18 (median 18.69 [IQR 16.73- 22.69]) using the
322 LightMix® assay. The relationship between C_t value and SARS-CoV-2 RNA copy numbers was
323 log-linear, with an $R^2 = 0.9990$ (Figure 6). Despite this strong relationship, inspection of the
324 residuals nevertheless suggested modest departures from log-linearity at the extremes of the
325 linear range (Supplementary Figure 4). The relationship between C_t value and absolute SARS-
326 CoV-2 E-gene copies can thus be given by $\log_{10}\text{SARS-CoV-2 E gene copies equivalent} =$
327 $-0.3038C_t + 11.7$ (Figure 6). That is, a C_t value of 20 corresponds to 453,942 (*i.e.* $5.66 \log_{10}$)
328 SARS-CoV-2 RNA copies, while a C_t value of 30 corresponds to 416 (*i.e.* $2.62 \log_{10}$) viral
329 copies. This equation also predicts that the C_t values corresponding to the LLOQ and LLOD of
330 the E-Sarbeco RT-ddPCR assays are 34.8 and 36.84, respectively. When measured SARS-CoV-2
331 RNA copy numbers are expressed as human cell-normalized viral loads, the relationship with C_t
332 value is given by $\log_{10}\text{SARS-CoV-2 E gene copies}/1,000 \text{ human cells} = -0.3041C_t + 10.8$
333 (Supplemental Figure 5). An extract that yielded a C_t value of 20 therefore is estimated to have
334 contained 48,978 (*i.e.* $4.69 \log_{10}$) SARS-CoV-2 RNA copies/1,000 human cells, while one with
335 C_t value of 30 is estimated to have contained 45 (*i.e.* $1.66 \log_{10}$) copies/1,000 human cells
336

337 **Discussion**

338 While real-time and droplet digital RT-PCR platforms both employ target-specific
339 primers coupled with fluorescence-based amplicon detection, there are key differences in
340 reaction chemistry (*e.g.* RT-ddPCR reagents must be compatible with water-in-oil droplet

341 partitioning) and probe chemistry (*e.g.* while real-time RT-PCR uses fluorescent quenchers,
342 ddPCR typically uses dark quenchers). As a result, assays developed for one platform may not
343 always translate seamlessly to the other. For example, ddPCR probes should ideally not have a
344 Guanine at their 5' end because this quenches the fluorescence signal even following hydrolysis
345 (36) but the HKU-N probe has a G at its 5' end (Table 1).

346 It is perhaps therefore not surprising that the overall performance of the eight
347 primer/probe sets in RT-ddPCR did not exactly mirror that in real-time RT-PCR (41, 42).
348 Nevertheless, E-Sarbeco, IP2 and IP4, which represented the most efficient and precise
349 primer/probe sets for SARS-CoV-2 RNA quantification by RT-ddPCR are also among the most
350 efficient in real-time RT-PCR (41, 42). Our results also confirm previous reports of the E-
351 Sarbeco primer/probe set performing well in RT-ddPCR (10, 22). Other primer/probe sets
352 however, notably US CDC-N1, HKU-ORF and China-ORF, did not perform as well in our RT-
353 ddPCR assay compared to a previous report (10). One key difference is that, while we used
354 sequence-specific reverse transcription (with the reverse primer) in a one-step RT-ddPCR
355 reaction, the previous study featured an independent reverse transcription reaction primed with
356 random hexamers and oligo dT, which can yield higher efficiency than sequence-specific
357 priming (35, 43-45), to generate cDNA for input into a ddPCR reaction. To our knowledge, ours
358 is the first study to evaluate IP2 or IP4 primer/probe sets in RT-ddPCR.

359 The analytical sensitivities of the RT-ddPCR assays reported here are nevertheless
360 comparable to existing estimates. The limit of detection of the BioRad SARS-CoV-2 ddPCR Kit
361 (20) is, for example, estimated at 150 copies/mL, which is comparable to our E-Sarbeco RT-
362 ddPCR assay (estimated at 75.8 copies/mL assuming 100% extraction efficiency). Similarly, the
363 LLODs of the TargetingOne (Beijing, China) COVID-19 digital PCR detection kit (23) and a

364 multiplex assay that included the E-Sarbeco primer/probe set (22) were reported at 10 copies/test
365 and 5 copies/reaction, respectively, both comparable to the LLOD determined here. While a
366 number of studies have reported that RT-ddPCR can detect SARS-CoV-2 RNA in low viral load
367 clinical samples with higher sensitivity than real-time RT-PCR (11, 21, 23-25), our study was
368 not designed to evaluate this. Our estimated LLOD of 4.4 copies/reaction by RT-ddPCR using
369 the E-Sarbeco primer/probe set (Figure 4) is in fact comparable to the LLOD reported for many
370 real-time RT-PCR-based COVID-19 diagnostic assays (46).

371 The ability to quantify SARS-CoV-2 viral loads in biological samples can advance our
372 understanding of COVID-19 biology, and RT-ddPCR offers an attractive platform (7, 8). Our
373 observation that, in a small convenience sample, both absolute and human cell-normalized (9)
374 SARS-CoV-2 loads spanned a more than 6 \log_{10} range confirms an enormous viral load range *in*
375 *vivo* (47) and suggests that some of the high viral load samples measured here were from
376 individuals with early and progressive infection (23, 48-50) or who were experiencing severe
377 disease (7, 8), though clinical information was unknown. Furthermore, our equation relating C_t
378 values derived from a commercial diagnostic assay and SARS-CoV-2 RNA copy number means
379 that existing diagnostic test results can be converted to viral loads *without re-testing samples*.
380 While calibration of viral load measurements against all real-time RT-PCR platforms is beyond
381 our scope, this is achievable and in some cases data may already be available (23).

382 Some limitations merit mention. We only tested eight commonly-used SARS-CoV-2-
383 specific primer/probe sets, and others may exist that adapt well to RT-ddPCR. Our assay
384 performance estimates should be considered approximate, as the manufacturer-reported
385 concentration of the synthetic SARS-CoV-2 RNA standards used in our study may vary by up to
386 20% error (Twist Bioscience, personal communication). Moreover, we solely evaluated a one-

387 step RT-ddPCR protocol, and therefore assay performance estimates will likely differ from
388 protocols that feature independent cDNA generation followed by ddPCR. We could not precisely
389 define the upper boundary of the linear dynamic range of the E-Sarbeco, IP2 and IP4 RT-ddPCR
390 assays as linearity was maintained at the maximum input of 114,286 target copies/reaction,
391 which already exceeds the manufacturer's estimated upper range of quantification in a ddPCR
392 reaction (36). Our convenience panel of 48 SARS-CoV-2-positive diagnostic specimens also
393 likely did not capture the full range of biological variation in viral loads, though data from larger
394 cohorts (47) suggests that it was reasonably comprehensive. We also acknowledge that there is
395 measurement uncertainty with real-time RT-PCR C_t values that may subtly affect the linear
396 relationship between C_t value and RT-ddPCR-derived SARS-CoV-2 viral load described here.
397 Finally, our estimates of assay performance may not completely reflect those of the entire
398 diagnostic process, as the nucleic acid extraction step introduces additional inefficiencies.

399 In conclusion, primer/probe sets used in real-time RT-PCR-based COVID-19 diagnostic
400 tests can be migrated to RT-ddPCR to achieve SARS-CoV-2 RNA quantification with varying
401 analytical efficiency, precision and sensitivity. Of the primer/probe sets tested, the E-Sarbeco,
402 IP2 and IP4 sets performed best, where LLOQ and LLOD estimates for the E-Sarbeco assay
403 (18.6 and 4.4 copies/reaction, respectively) indicated that RT-ddPCR and real-time RT-PCR
404 have comparable sensitivity. Mathematical inference of SARS-CoV-2 copy numbers from
405 COVID-19 diagnostic test C_t values, made possible via the type of calibration performed in the
406 present study, will allow the wealth of existing diagnostic test data to be harnessed to answer
407 foundational questions in SARS-CoV-2 biology.

408

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Source	Name	Gene Target	Primer/ Probe	Sequence ^φ (5'→3')	Coordinates ^ψ
Charité-Berlin	E-Sarbeco	E	Fwd Primer	ACAGGTACGTTAATAGTTAATAGCGT	26,269- 26,294
			Rev Primer	ATATTGCAGCAGTACGCACACA	26,381- 26,360
			Probe	FAM-ACACTAGCC/ZEN/ATCCTTACTGCGCTTCG-3IABkFQ	26,332- 26,357
Pasteur Institute	IP2	ORF1a	Fwd Primer	ATGAGCTTAGTCCTGTTG	12,690- 12,707
			Rev Primer	CTCCCTTTGTTGTGTTGT	12,797- 12,780
			Probe	HEX-AGATGTCTT/ZEN/GTGCTGCCGGTA-3IABkFQ	12,717- 12,737
	IP4	ORF1b	Fwd Primer	GGTAACTGGTATGATTTTCG	14,080- 14,098
			Rev Primer	CTGGTCAAGGTTAATATAGG	14,105- 14,123
			Probe	FAM-TCATACAAA/ZEN/CCACGCCAGG-3IABkFQ	14,186- 14,167
China CDC	China-ORF	ORF1a	Fwd Primer	CCCTGTGGGTTTTACACTTAA	13,342- 13,362
			Rev Primer	ACGATTGTGCATCAGCTGA	13,460- 13,442
			Probe	FAM-CCGTCTGCG/ZEN/GTATGTGGAAAGGTTATGG-3IABkFQ	13,377- 13,404
	China-N	N	Fwd Primer	GGGGAACTTCTCCTGCTAGAAT	28,881- 28,902
			Rev Primer	CAGACATTTTGCTCTCAAGCTG	28,979- 28,958
			Probe	FAM-TTGCTGCTG/ZEN/CTTGACAGATT-3IABkFQ	28,934- 28,953
Hong Kong University	HKU-ORF	ORF1b	Fwd Primer	TGGGGYTTTACRGGTAACT	18,778- 18,797
			Rev Primer	AACRCGCTTAACAAAGCACTC	18,849- 18,872
			Probe	FAM-TAGTTGTGA/ZEN/TGCWATCATGACTAG-3IABkFQ	18,909- 18,889
	HKU-N	N	Fwd Primer	TAATCAGACAAGGAAGTATTA	29,145- 29,166
			Rev Primer	CGAAGGTGTGACTTCCATG	29,179- 29,198
			Probe	FAM-GCAAATTGT/ZEN/GCAATTTGCGG-3IABkFQ	29,254- 29,236
US CDC	US-CDC-N1	N	Fwd Primer	GACCCCAAATCAGCGAAAT	28,287- 28,306
			Rev Primer	TCTGGTACTGCCAGTTGAATCTG	28,358- 28,335
			Probe	FAM-ACCCCGCAT/ZEN/TACGTTTGGTGGACC-3IABkFQ	28,309- 28,332

^φ FAM= 6-Carboxyfluorescein; HEX= Hexachloro-Fluorescein; ZEN= internal ZEN quencher (Integrated DNA Technologies); 3IABkFQ= 3' Iowa Black Black Hole Quencher (Integrated DNA Technologies)

^ψ Coordinates based on the SARS-CoV-2 Wuhan-Hu-1 genome (Genbank Accession Number: MN908947.3)

Figure 1: Thermal cycling optimization (A). RT-ddPCR plots for annealing/extension under a 50-63°C thermal gradient for the E-Sarbeco primer/probe set. A representative RT-ddPCR plot for a no template control (NTC) which only included non-target DNA/RNA (see methods) at the temperature used in subsequent experiments, is also shown. Positive droplets (blue) are above the threshold (pink line); negative droplets (grey) are below the line. Colored boxes below each well indicate if results met standards for inclusion (green) or not (red) (see methods). (B). Same as panel A, but for HKU-ORF primer/probe set. (C). Acceptable RT and annealing/extension temperature ranges for each primer/probe set.

Figure 2: Analytical efficiency and precision of primer/probe sets. (A) Analytical efficiency of each primer/probe set, calculated as the measured divided by the input SARS-CoV-2 RNA copies multiplied by 100%, is shown for reactions containing 1,000 and 100 input copies of synthetic SARS-CoV-2 RNA. Bars represent 95% Total Poisson Confidence Intervals. (B). Precision of each primer/probe set, defined as the coefficient of variation (expressed as a percentage, CV%) of measured copies, is shown for reactions containing 1,000 and 100 input copies of synthetic SARS-CoV-2 RNA. (C). Plotting precision versus analytical efficiency at 1,000 input SARS-CoV-2 RNA copies identifies E-Sarbeco, IP2 and IP4 primer/probe sets as having analytical efficiencies >50% and CV (%) <15%. (D). Same as C, but for 100 input SARS-CoV-2 RNA copies.

Figure 3: Linear Dynamic Range (LDR) of E-Sarbeco, IP2 and IP4 RT-ddPCR assays. (A). left: \log_{10} Measured SARS-CoV-2 RNA copies over serial dilutions of synthetic SARS-CoV-2 RNA standards ranging from 114,286 to 2.32 copies/reaction (shown as \log_{10} values), using the

E-Sarbeco primer/probe set. Error bars indicate 95% Total Poisson Confidence Intervals for two merged replicates, where in some cases error bars are too small to visualize. The regression line joins all data points included in the LDR, where the lower boundary of the LDR represents the lower limit of quantification (LLOQ) of the assay. Data points that yielded undetectable measurements are set arbitrarily to $-0.35 \log_{10} \text{Measured copies/reaction}$ for visualization. right: $\log_{10} \text{Residuals}$, calculated as $\log_{10} \text{Measured SARS-CoV-2 RNA copies/reaction}$ minus $\log_{10} \text{Calculated SARS-CoV-2 RNA copies/reaction}$ from the LDR regression. Grey shading indicates data points outside the LDR. Residuals for data points that yielded undetectable measurements are arbitrarily set to -0.4 for visualization. (B). Same as A, but for the IP2 primer/probe set (C). Same as A, but for the IP4 primer/probe set.

Figure 4: Lower Limit of Detection (LLOD) of the E-Sarbeco, IP2 and IP4 RT-ddPCR assays. (A). The probability of detecting SARS-CoV-2 RNA (%) in 1:2 in serial dilutions of synthetic SARS-CoV-2 RNA from 47.6 to 0.74 input copies/reaction using the E-Sarbeco primer/probe set is analyzed using probit regression (solid black line; dashed line denotes the 95% confidence interval). The LLOD, defined as the concentration of SARS-CoV-2 RNA in a reaction where the probability of detection in the assay was 95%, was interpolated from the standard curve and is shown as a colored dashed line (B). Same as A, but for the IP2 primer/probe set (C). Same as A, but for the IP4 primer/probe set.

Figure 5: \log_{10} SARS-CoV-2 RNA loads in diagnostic specimens (A). SARS-CoV-2 E (green circles), ORF1a (red squares) and ORF1b (blue triangles) gene copy numbers, expressed as RNA copies/ μl of nucleic acid extract. Line and bars indicate median and interquartile range,

respectively. **(B)** Correlation between Log_{10} SARS-CoV-2 E and ORF1a gene RNA copies/ μl extract. **(C)** Correlation between Log_{10} SARS-CoV-2 E and ORF1b gene RNA copies/ μl extract. **(D)** Correlation and Concordance between Log_{10} SARS-CoV-2 ORF1a and ORF1b gene RNA copies/ μl extract.

Figure 6: Relationship between SARS-CoV-2 RNA copies equivalent and diagnostic test C_t value. C_t value, determined using the LightMix® 2019-nCoV real-time RT-PCR assay (E-gene target) is plotted against log_{10} SARS-CoV-2 E gene RNA copies equivalent, which represents the number of SARS-CoV-2 RNA copies measured by RT-ddPCR in 9 μl extract (the template volume in the LightMix® assay). The linear regression (solid black line) transitions to a dashed line below the LLOQ.

Supplementary Figure 1: All experiments using synthetic SARS-CoV-2 synthetic standards were performed in a consistent background of human nucleic acids to mimic a real human sample. Example experiment showing consistent levels of background human cells/ μl extract (determined by dividing measured human RPP30 DNA copy number by two; black triangles), and human RNase P RNA levels (grey squares) across a titration of SARS-CoV-2 synthetic RNA standards, measured using the E-Sarbeco primer/probe set (green circles). Error bars indicate 95% Total Poisson Confidence Intervals for two merged replicates, where in some cases error bars are too small to visualize. Grey (RNase P) and black (RPP30) dashed lines indicate copies measured control experiments lacking SARS-CoV-2 RNA.

Supplementary Figure 2: Duplexing the IP2 and IP4 primer/probe sets reduces analytical

efficiency and precision. (A). Analytical efficiency of SARS-CoV-2 quantification was evaluated for the IP2 and IP4 primer/probe sets when used in separate reactions (dark red and dark blue, respectively) and when duplexed (light red and light blue, respectively), in reactions containing 1,000 and 100 viral RNA input copies. Error bars represent 95% Total Poisson Confidence Intervals. (B). Same as A, but for assay precision (coefficient of variation, CV%).

Supplementary Figure 3: Log₁₀SARS-CoV-2 RNA loads in diagnostic specimens,

normalized to human cells sampled. (A) SARS-CoV-2 E (green circles), ORF1a (red squares) and ORF1b (blue triangles) gene copy numbers, expressed as RNA copies/1,000 human cells. Line and bars indicate median and interquartile range, respectively. (B) Correlation between SARS-CoV-2 RNA copies/ μ l extract and RNA copies/1,000 human cells.

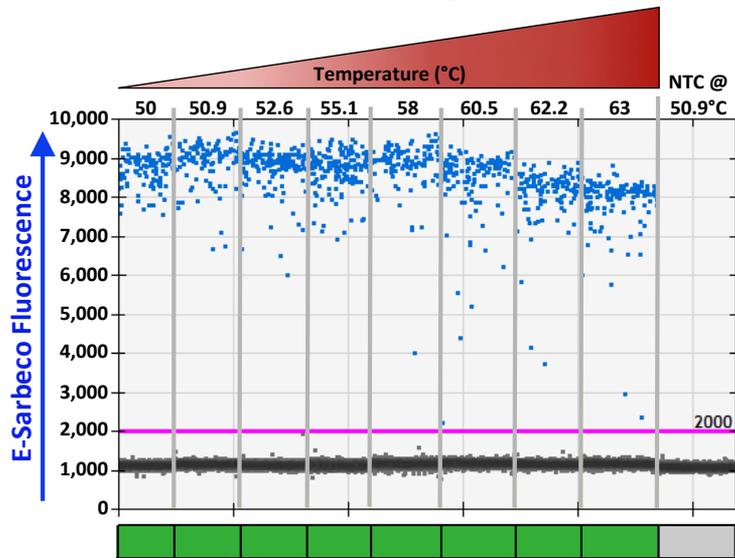
Supplemental Figure 4: Residuals of relationship between SARS-CoV-2 RNA copies

equivalent and diagnostic test C_t value. Log₁₀Residuals are calculated as log₁₀Measured SARS-CoV-2 RNA copies equivalent minus log₁₀Calculated SARS-CoV-2 RNA copies equivalent from the regression line shown in Figure 6.

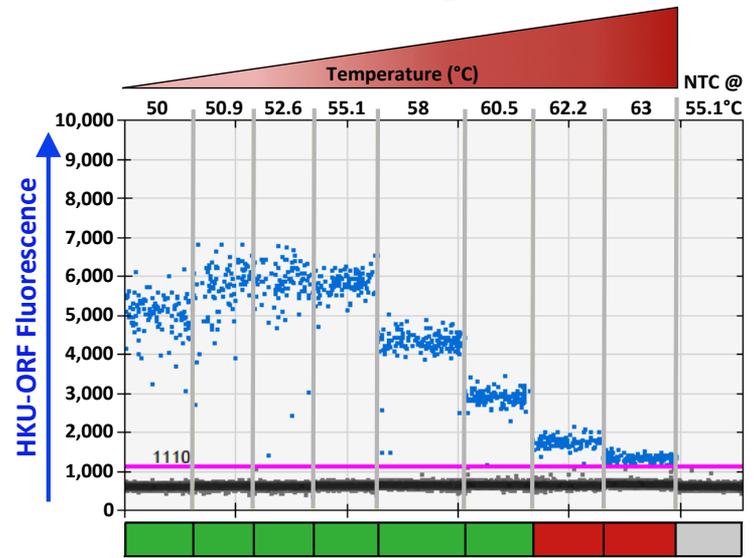
Supplemental Figure 5: Relationship between SARS-CoV-2 RNA copies/1,000 human cells

and C_t value. Same data as shown in Figure 6, but where the measured SARS-CoV-2 RNA copies/ μ l extract were normalized to copies/1,000 human cells. The linear regression is shown as a solid black line.

A E-Sarbeco Annealing/Extension



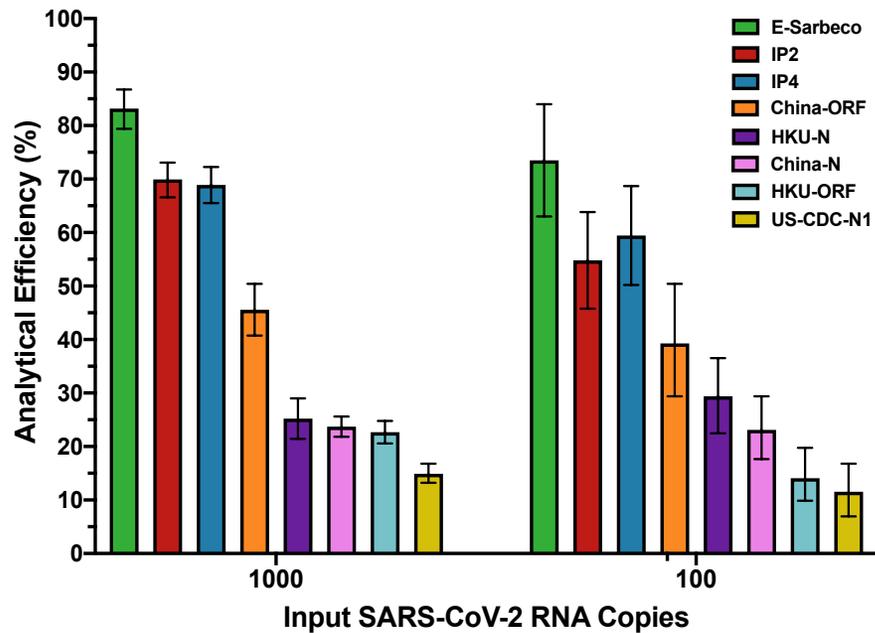
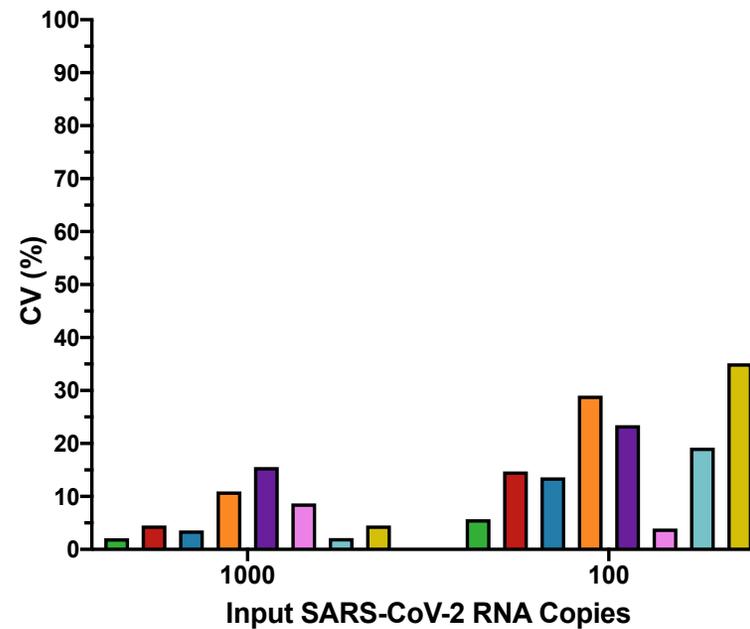
B HKU-ORF Annealing/Extension



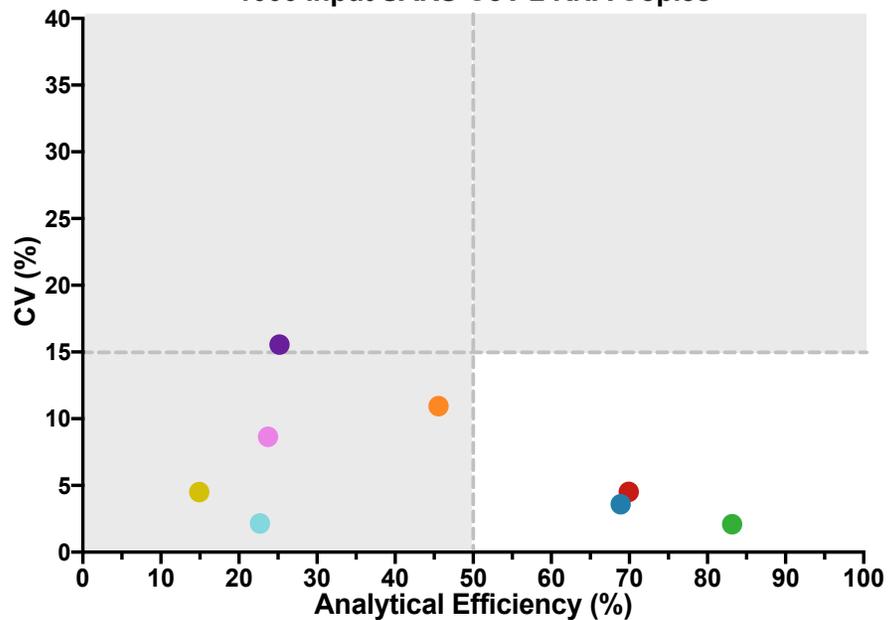
C

Acceptable Temperature °C	E-Sarbeco	IP2	IP4	CHINA-ORF	CHINA-N	HKU-ORF	HKU-N	US-CDC-N1
RT	42- 49.7	42- 51.5	42- 50.9	42- 51.5	42.7- 50.9	42- 51.5	42- 51.5	42- 45.7
Annealing/Extension	50- 63	50- 60.5	50- 60.5	50- 63	50- 60.5	50- 60.5	50.9- 60.5	50- 63

Figure 1: Thermal cycling optimization (A). RT-ddPCR plots for annealing/extension under a 50-63°C thermal gradient for the E-Sarbeco primer/probe set. A representative RT-ddPCR plot for a no template control (NTC) which only included non-target DNA/RNA (see methods) at the temperature used in subsequent experiments, is also shown. Positive droplets (blue) are above the threshold (pink line); negative droplets (grey) are below the line. Colored boxes below each well indicate if results met standards for inclusion (green) or not (red) (see methods). (B). Same as panel A, but for HKU-ORF primer/probe set. (C). Acceptable RT and annealing/extension temperature ranges for each primer/probe set.

A**B****C**

1000 Input SARS-CoV-2 RNA Copies

**D**

100 Input SARS-CoV-2 RNA Copies

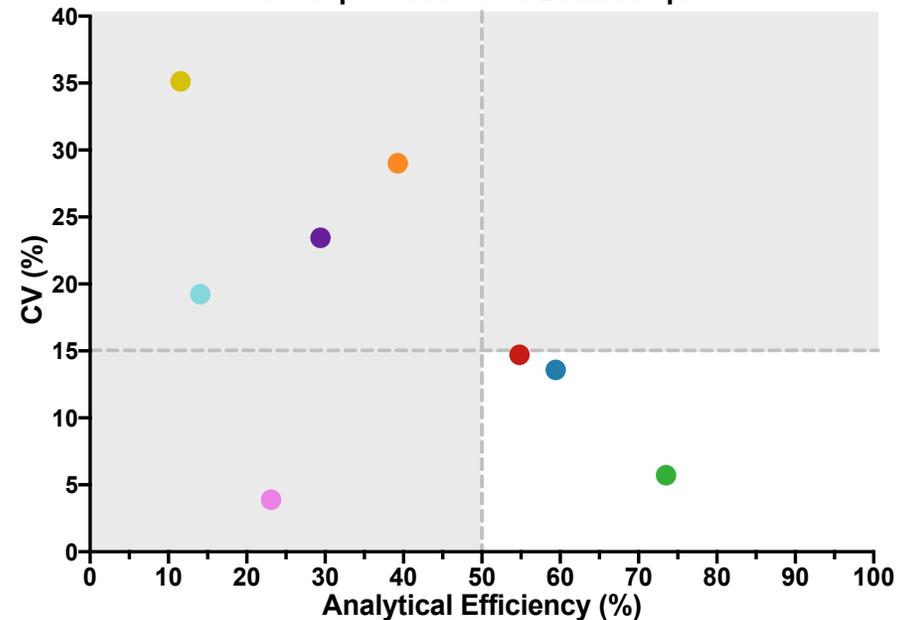


Figure 2: Analytical efficiency and precision of primer/probe sets. (A) Analytical efficiency of each primer/probe set, calculated as the measured divided by the input SARS-CoV-2 RNA copies multiplied by 100%, is shown for reactions containing 1,000 and 100 input copies of synthetic SARS-CoV-2 RNA. Bars represent 95% Total Poisson Confidence Intervals. (B). Precision of each primer/probe set, defined as the coefficient of variation (expressed as a percentage, CV%) of measured copies, is shown for reactions containing 1,000 and 100 input copies of synthetic SARS-CoV-2 RNA. (C). Plotting precision versus analytical efficiency at 1,000 input SARS-CoV-2 RNA copies identifies E-Sarbeco, IP2 and IP4 primer/probe sets as having analytical efficiencies >50% and CV (%) <15%. (D). Same as C, but for 100 input SARS-CoV-2 RNA copies.

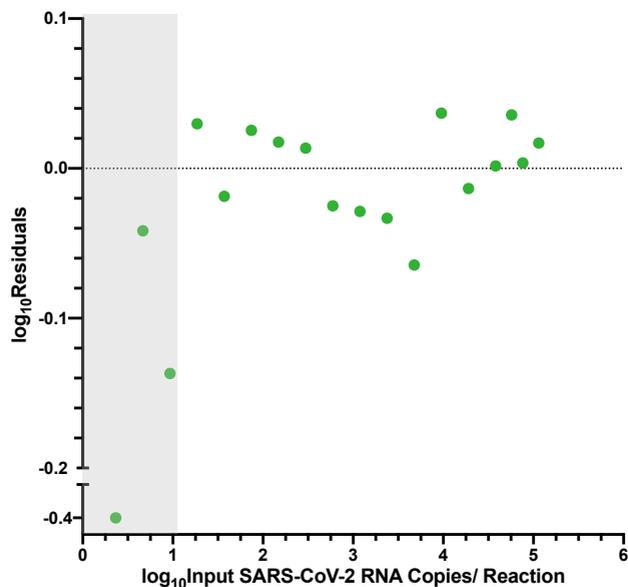
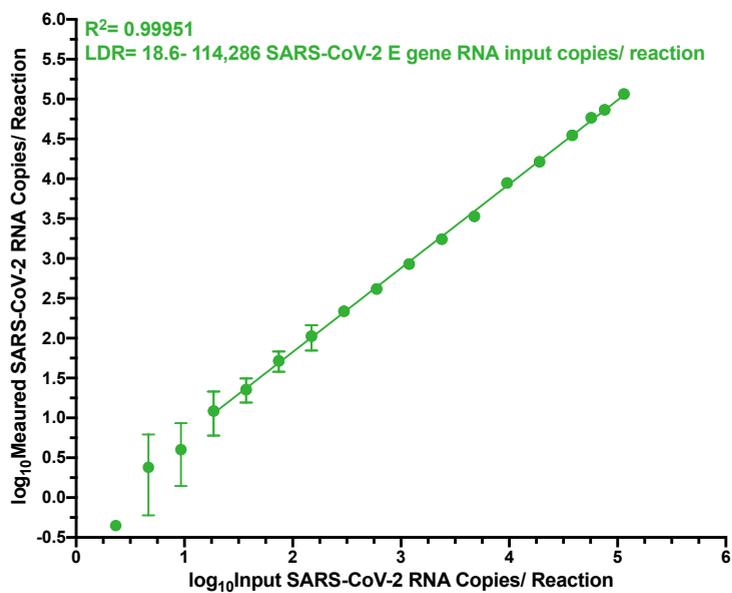
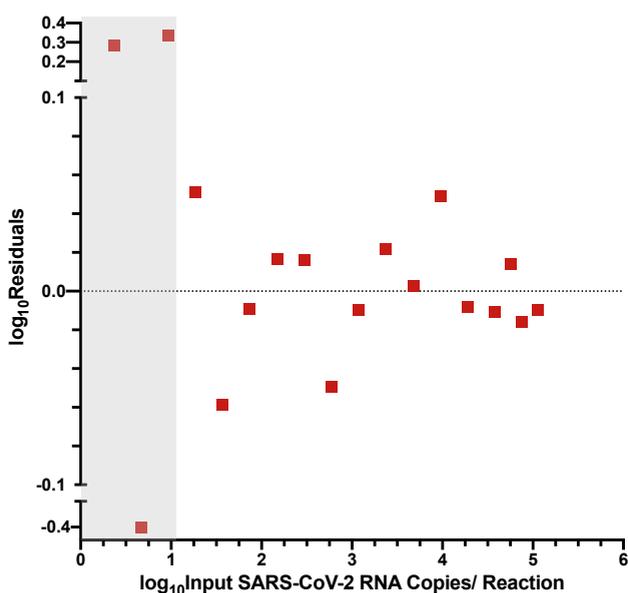
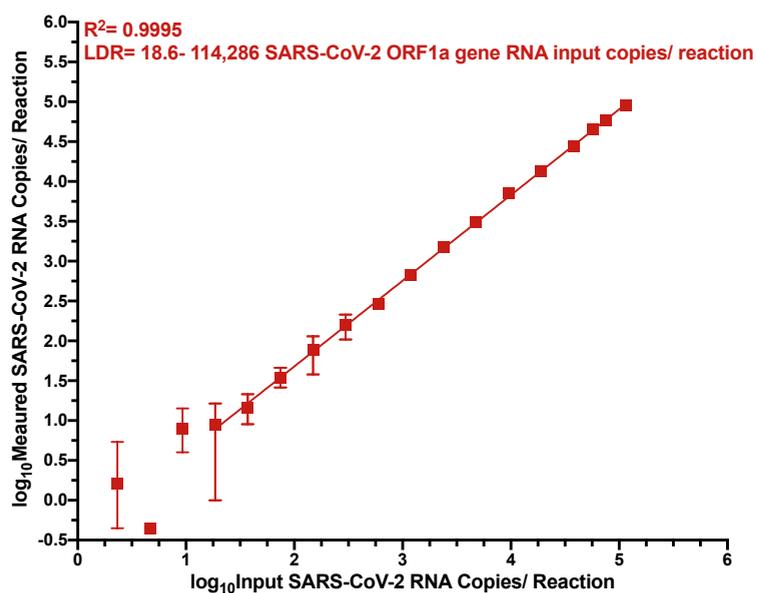
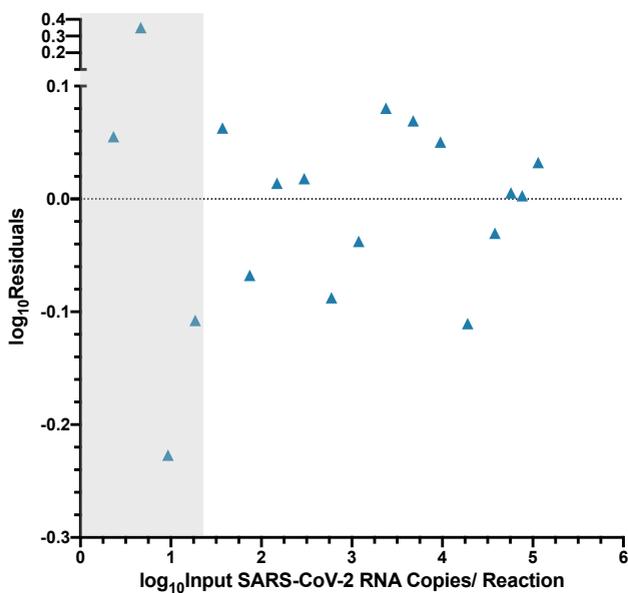
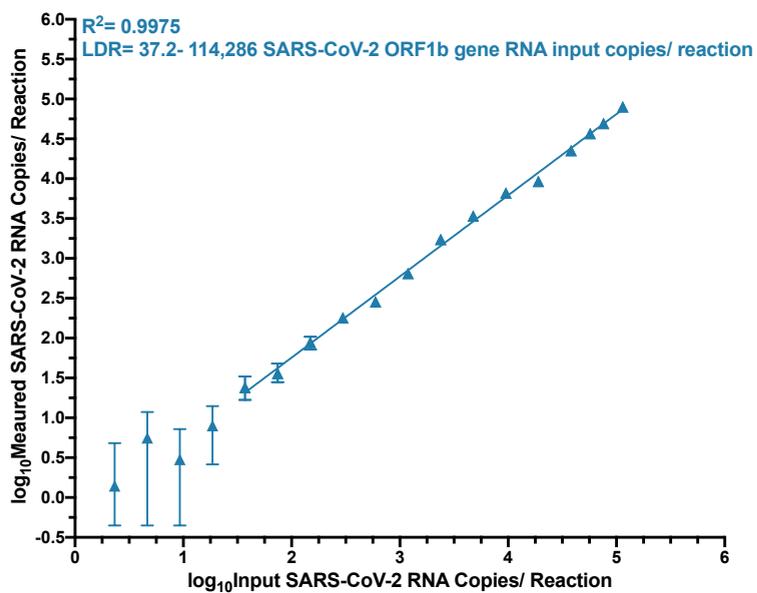
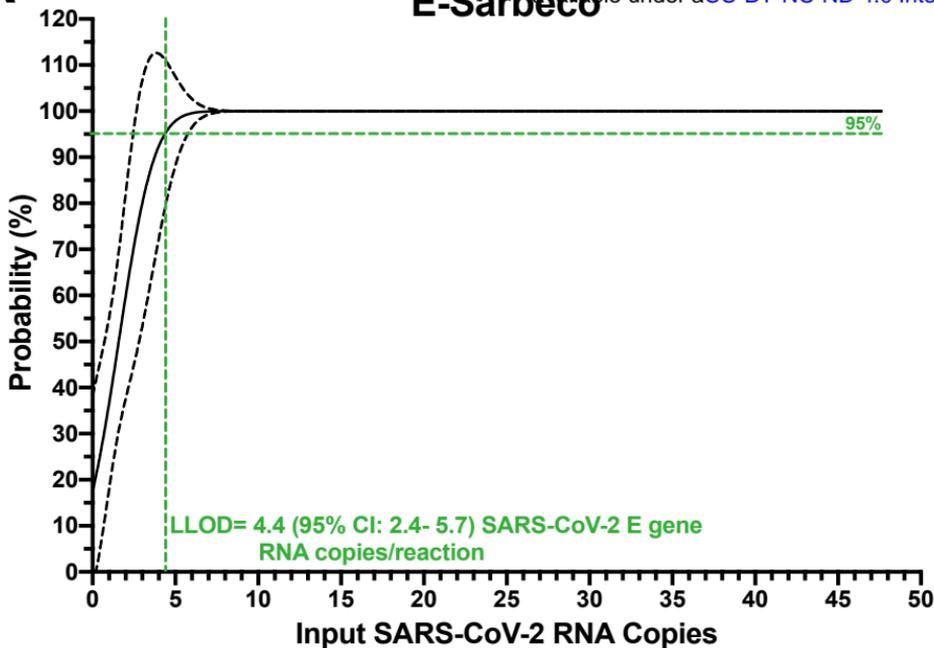
A**E-Sarbeco****B****IP2****C****IP4**

Figure 3: Linear Dynamic Range (LDR) of E-Sarbeco, IP2 and IP4 RT-ddPCR assays. (A). left: \log_{10} Measured SARS-CoV-2 RNA copies over serial dilutions of synthetic SARS-CoV-2 RNA standards ranging from 114,286 to 2.32 copies/reaction (shown as \log_{10} values), using the E-Sarbeco primer/probe set. Error bars indicate 95% Total Poisson Confidence Intervals for two merged replicates, where in some cases error bars are too small to visualize. The regression line joins all data points included in the LDR, where the lower boundary of the LDR represents the lower limit of quantification (LLOQ) of the assay. Data points that yielded undetectable measurements are set arbitrarily to $-0.35 \log_{10}$ Measured copies/reaction for visualization. right: \log_{10} Residuals, calculated as \log_{10} Measured SARS-CoV-2 RNA copies/reaction minus \log_{10} Calculated SARS-CoV-2 RNA copies/reaction from the LDR regression. Grey shading indicates data points outside the LDR. Residuals for data points that yielded undetectable measurements are arbitrarily set to -0.4 for visualization. (B). Same as A, but for the IP2 primer/probe set (C). Same as A, but for the IP4 primer/probe set.

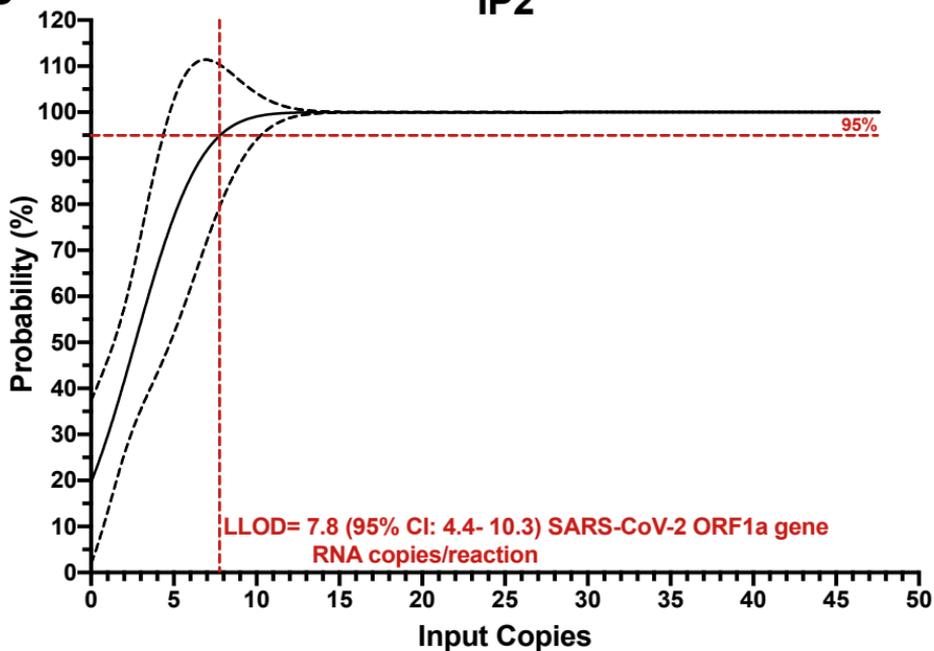
A

E-Sarbeco



B

IP2



C

IP4

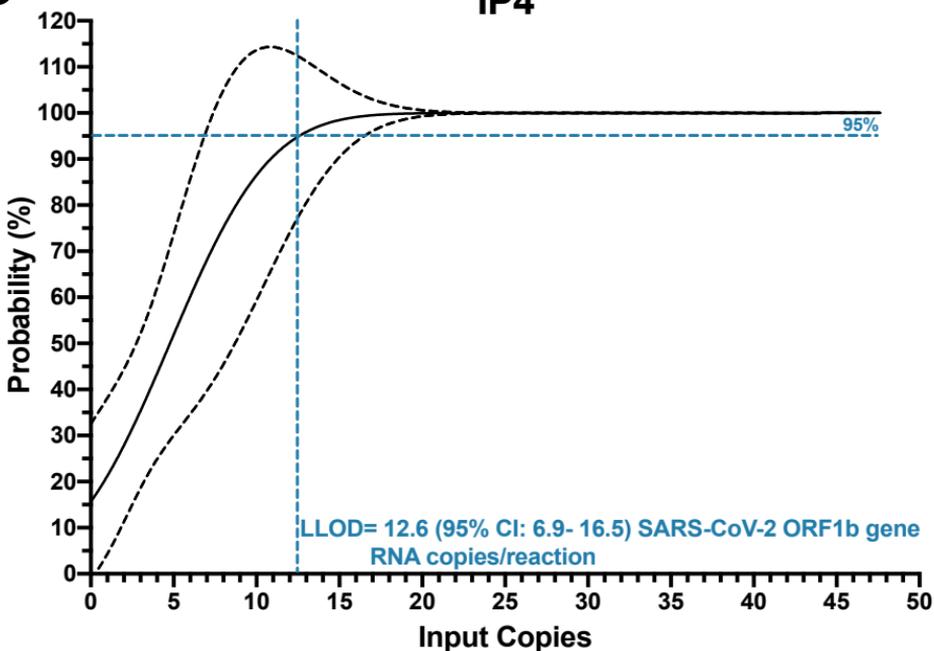


Figure 4: Lower Limit of Detection (LLOD) of the E-Sarbeco, IP2 and IP4 RT-ddPCR assays. (A). The probability of detecting SARS-CoV-2 RNA (%) in 1:2 in serial dilutions of synthetic SARS-CoV-2 RNA from 47.6 to 0.74 input copies/reaction using the E-Sarbeco primer/probe set is analyzed using probit regression (solid black line; dashed line denotes the 95% confidence interval). The LLOD, defined as the concentration of SARS-CoV-2 RNA in a reaction where the probability of detection in the assay was 95%, was interpolated from the standard curve and is shown as a colored dashed line (B). Same as A, but for the IP2 primer/probe set (C). Same as A, but for the IP4 primer/probe set.

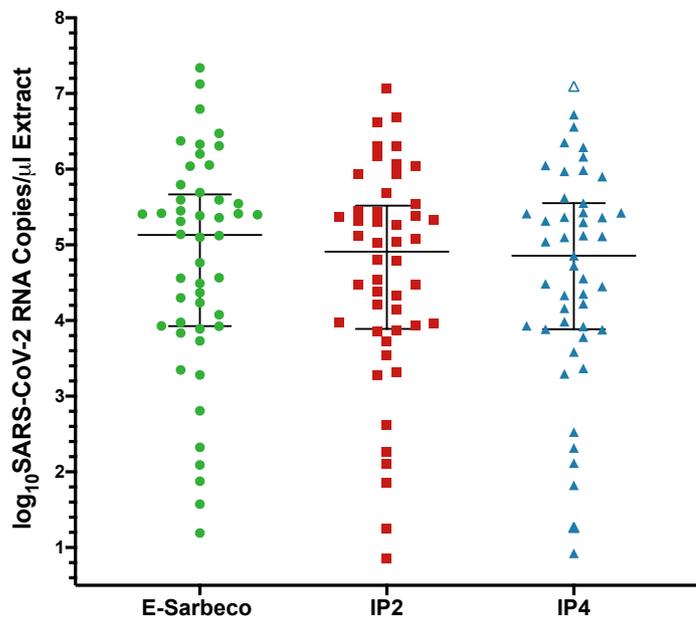
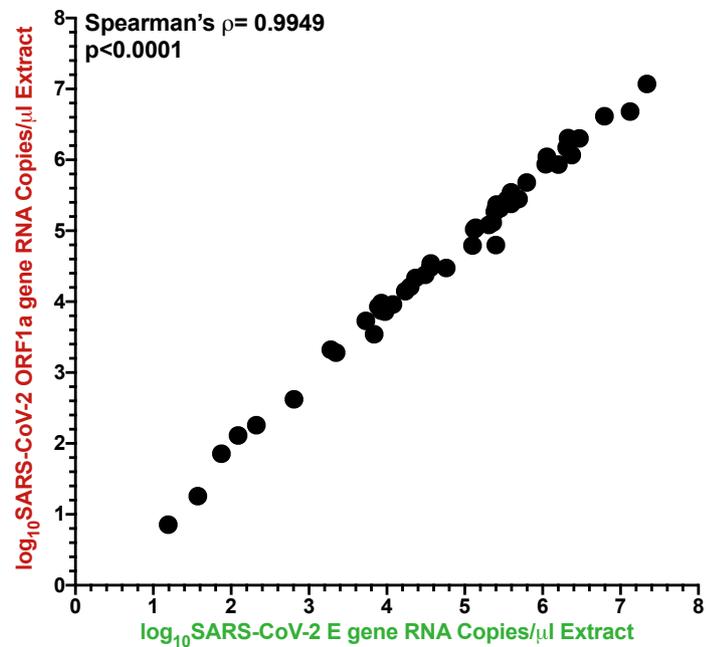
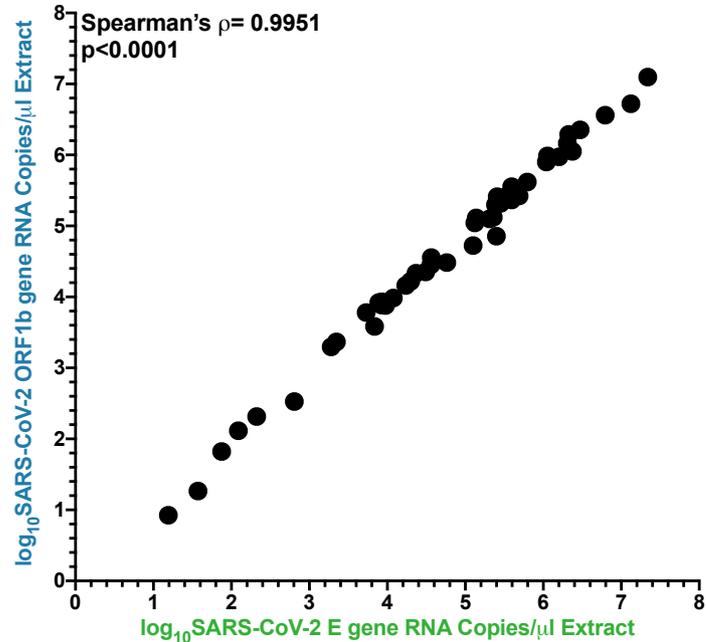
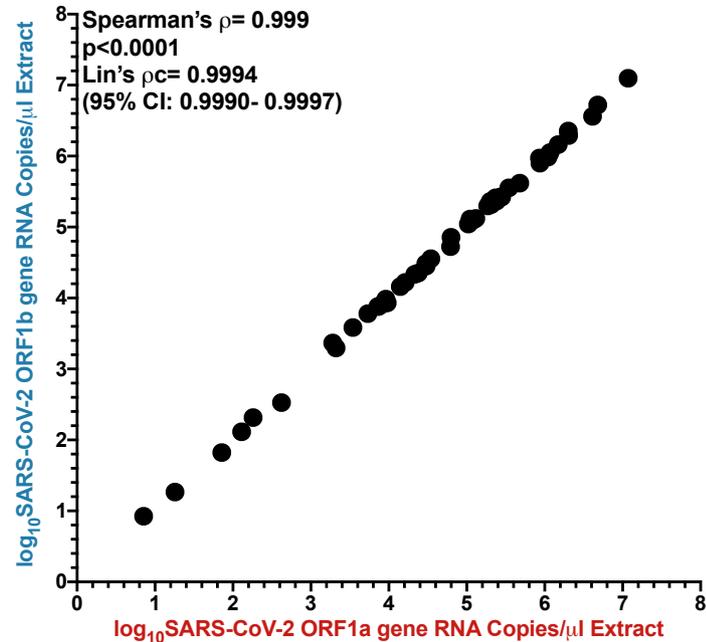
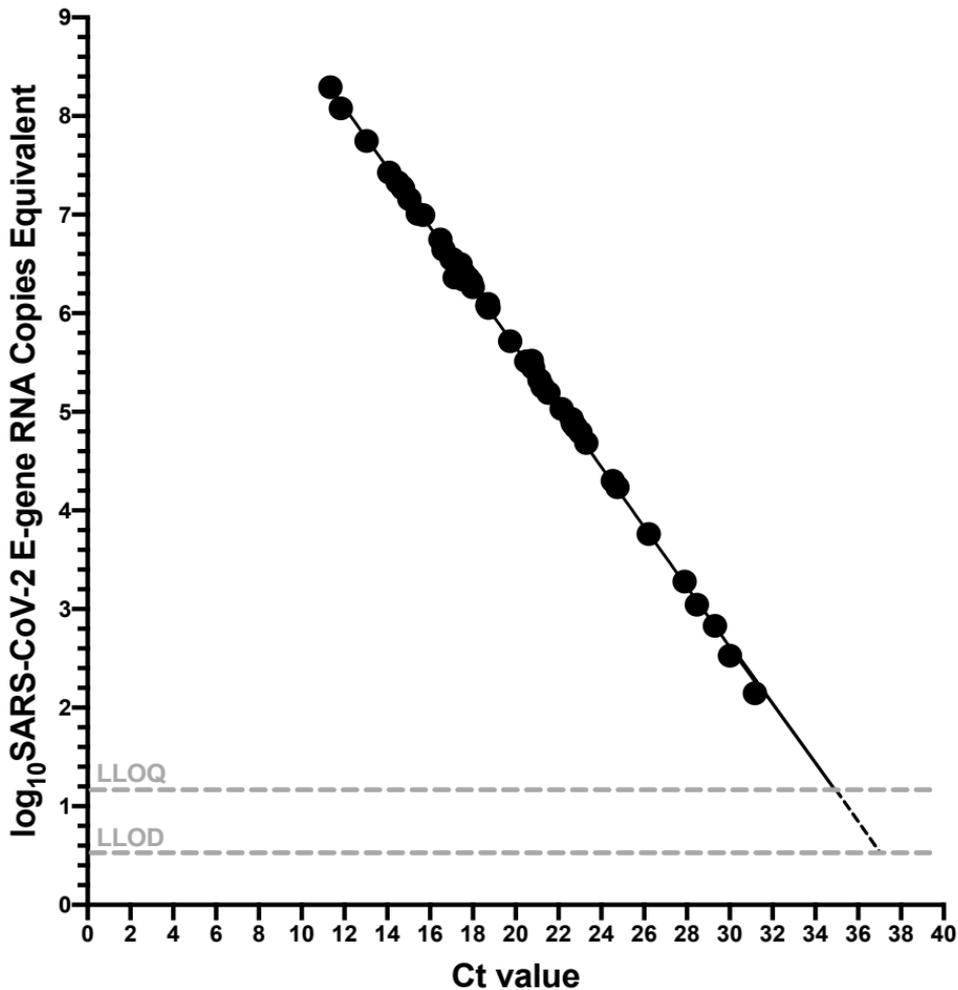
A**B****C****D**

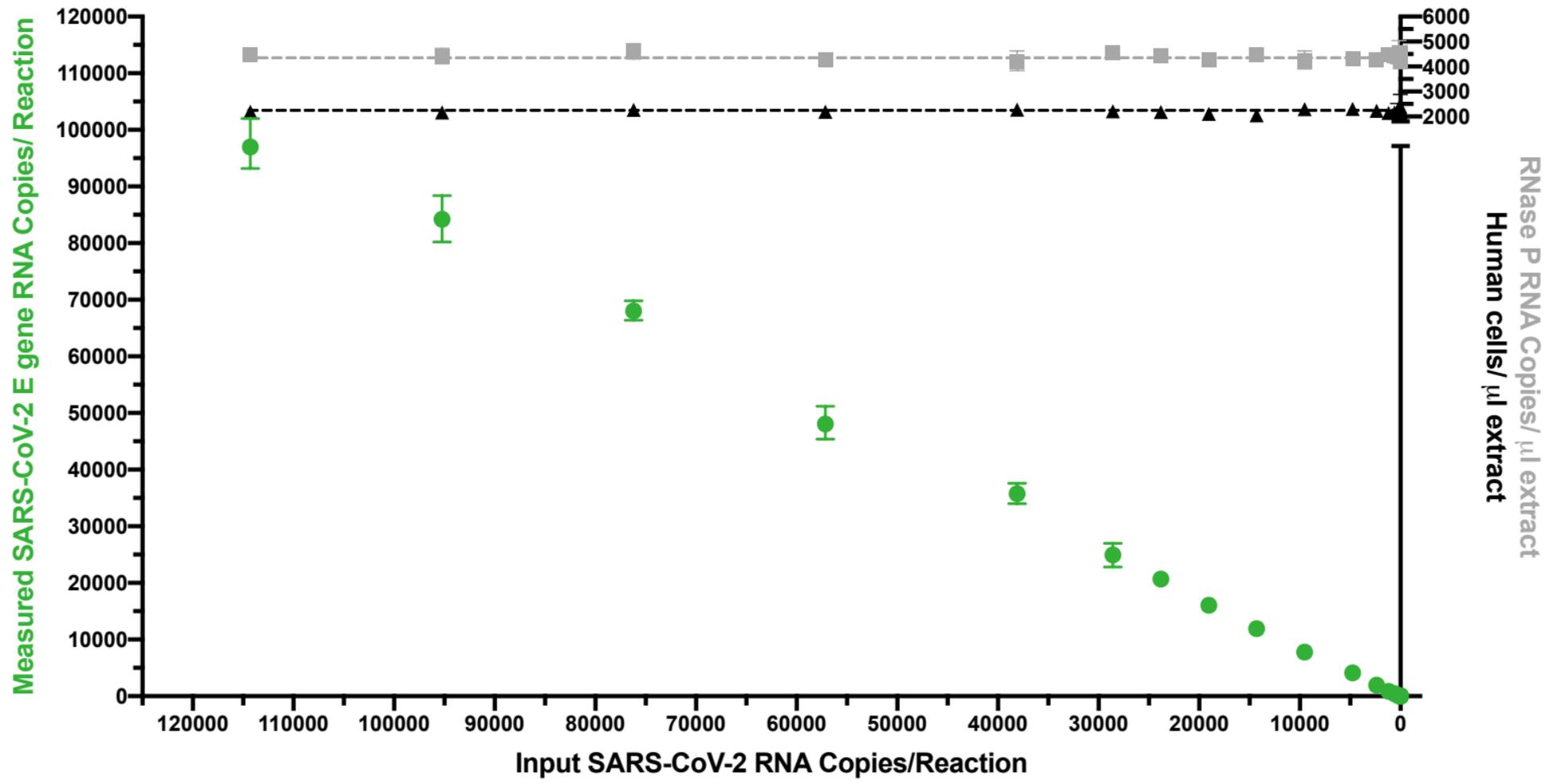
Figure 5: Log₁₀SARS-CoV-2 RNA loads in diagnostic specimens (A). SARS-CoV-2 E (green circles), ORF1a (red squares) and ORF1b (blue triangles) gene copy numbers, expressed as RNA copies/ μ l of nucleic acid extract. Line and bars indicate median and interquartile range, respectively. **(B)** Correlation between Log₁₀SARS-CoV-2 E and ORF1a gene RNA copies/ μ l extract. **(C).** Correlation between Log₁₀SARS-CoV-2 E and ORF1b gene RNA copies/ μ l extract. **(D)** Correlation and Concordance between Log₁₀SARS-CoV-2 ORF1a and ORF1b gene RNA copies/ μ l extract.



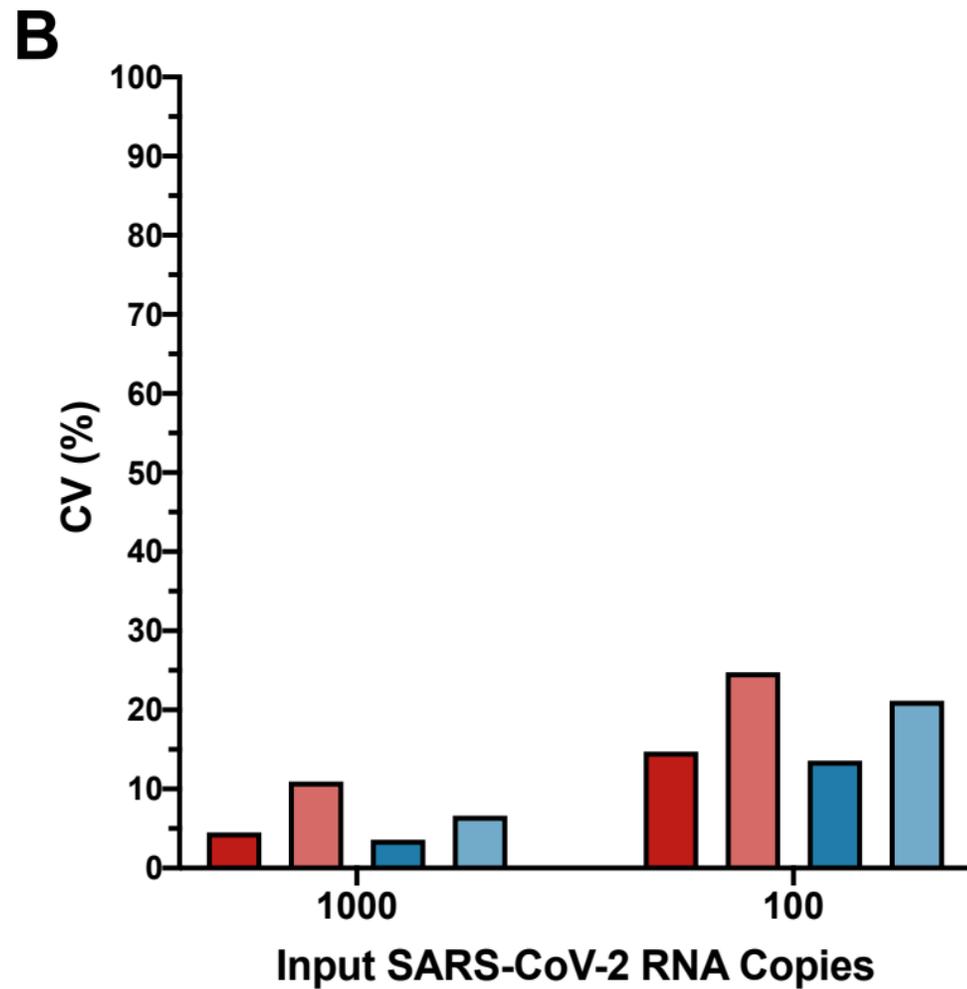
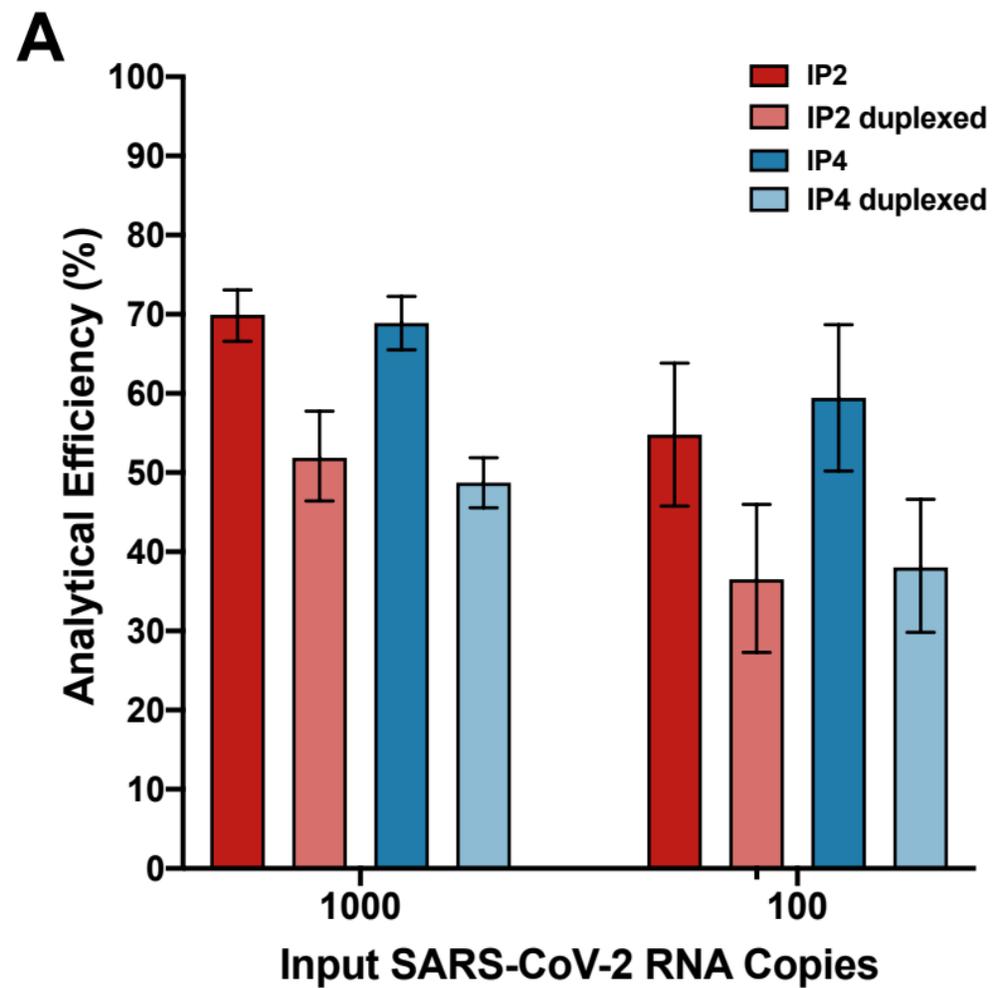
$R^2 = 0.99901$

$\log_{10} \text{SARS-CoV-2 E-gene RNA Copies Equivalent} = -0.3038(C_t) + 11.7$

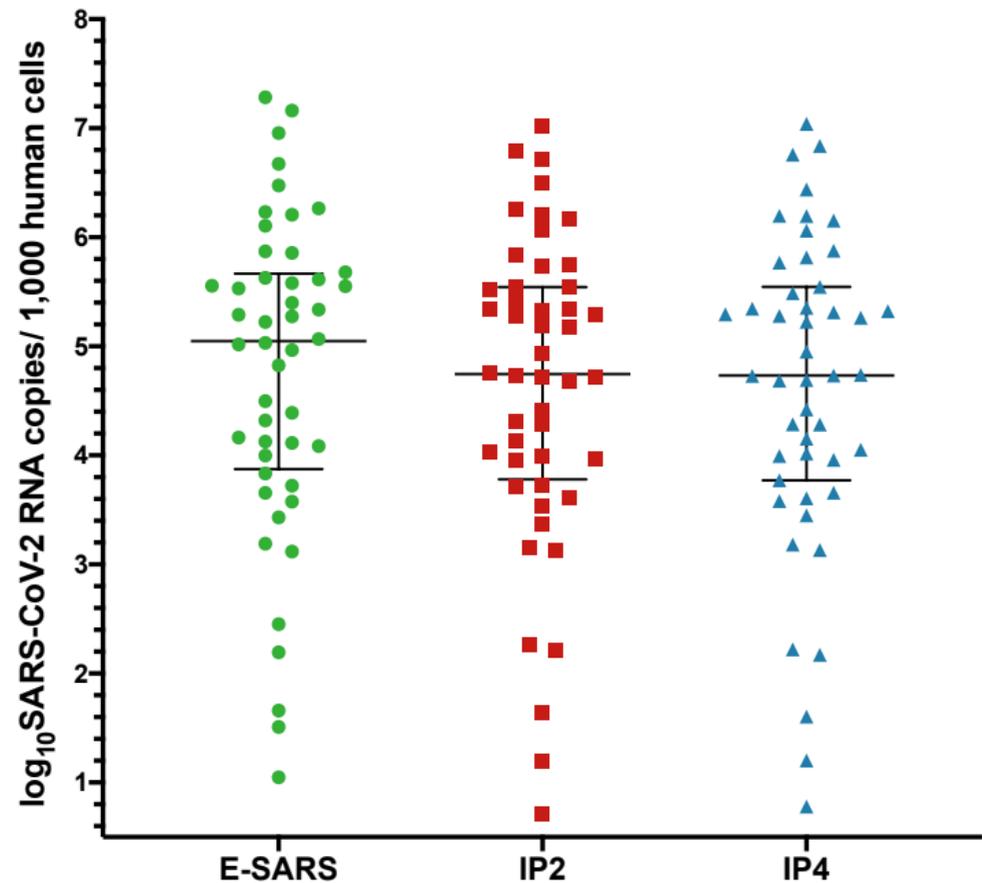
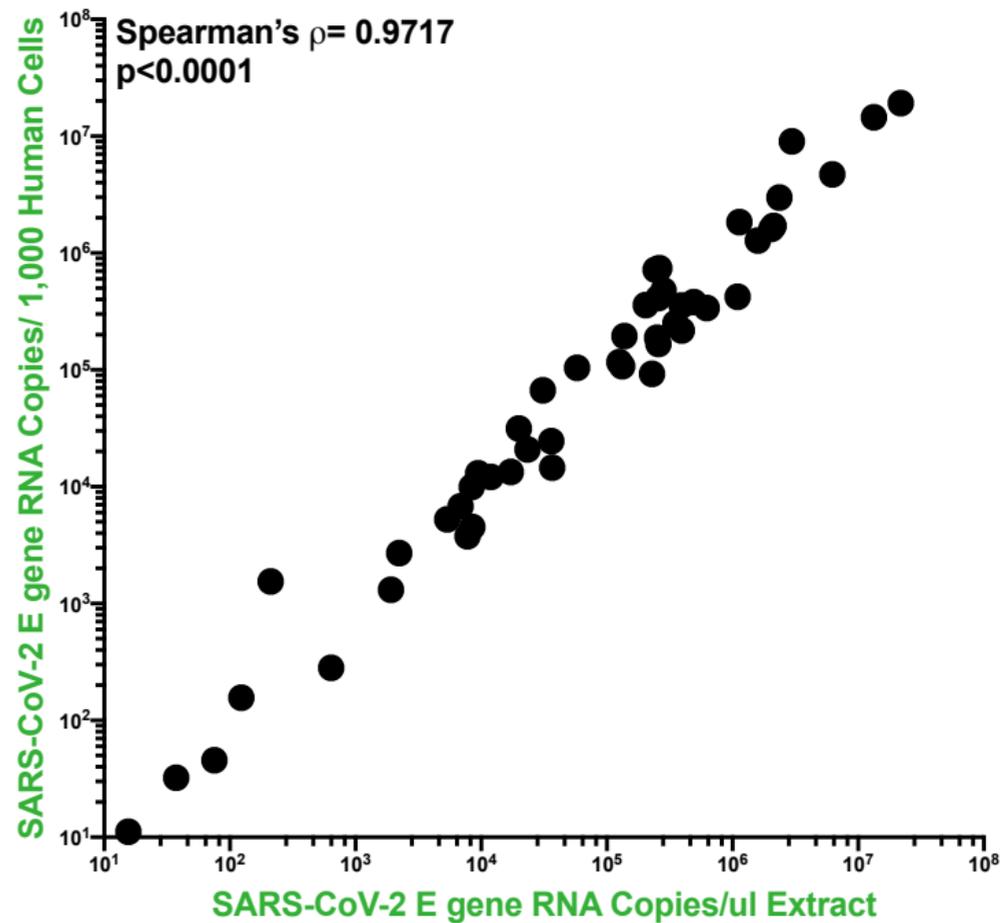
Figure 6: Relationship between SARS-CoV-2 RNA copies equivalent and diagnostic test C_t value. C_t value, determined using the LightMix® 2019-nCoV real-time RT-PCR assay (E-gene target) is plotted against \log_{10} SARS-CoV-2 E gene RNA copies equivalent, which represents the number of SARS-CoV-2 RNA copies measured by RT-ddPCR in 9 μ l extract (the template volume in the LightMix® assay). The linear regression (solid black line) transitions to a dashed line below the LLOQ.



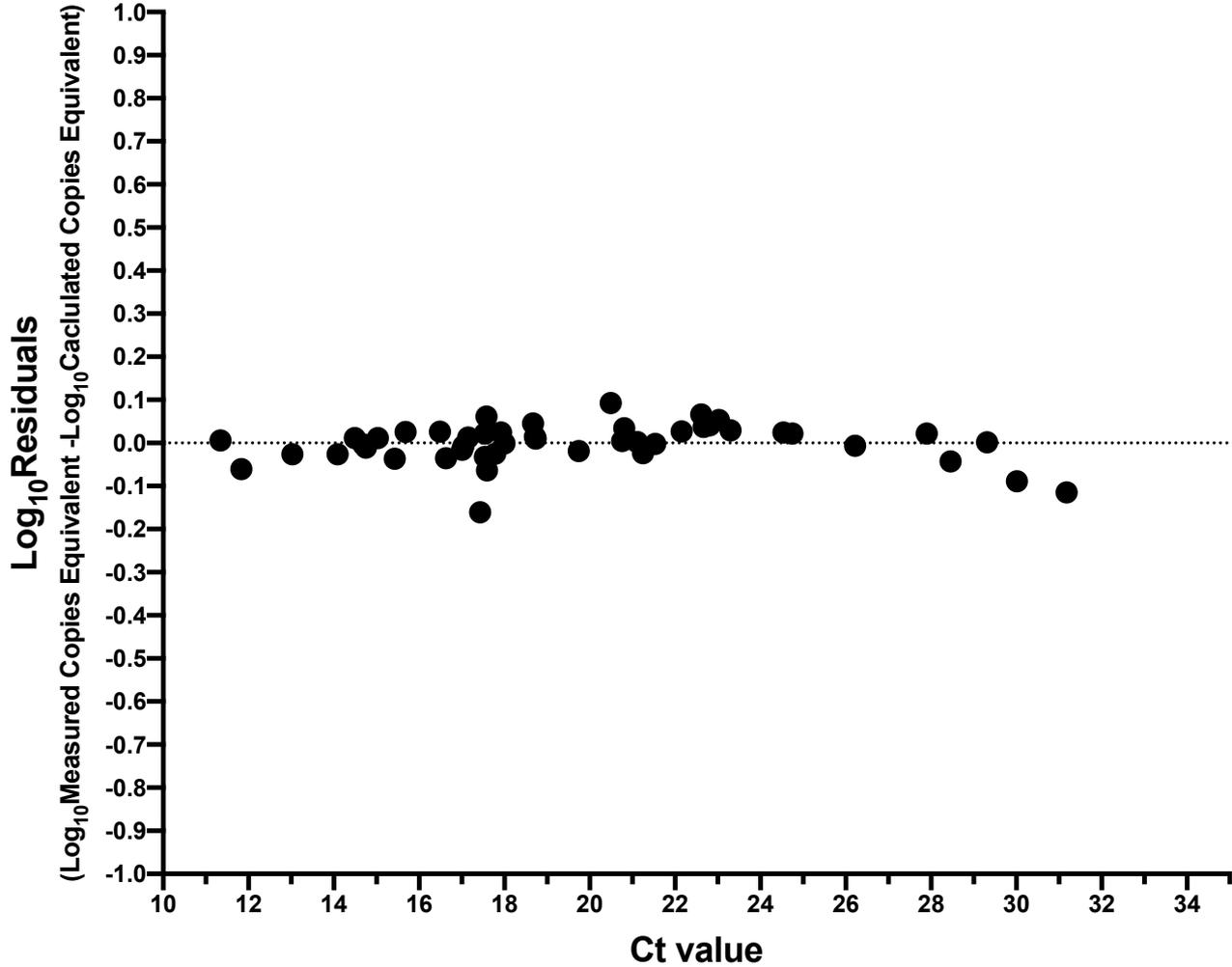
Supplementary Figure 1: All experiments using synthetic SARS-CoV-2 synthetic standards were performed in a consistent background of human nucleic acids to mimic a real human sample. Example experiment showing consistent levels of background human cells/ μl extract (determined by dividing measured human RPP30 DNA copy number by two; black triangles), and human RNase P RNA levels (grey squares) across a titration of SARS-CoV-2 synthetic RNA standards, measured using the E-Sarbeco primer/probe set (green circles). Error bars indicate 95% Total Poisson Confidence Intervals for two merged replicates, where in some cases error bars are too small to visualize. Grey (RNase P) and black (RPP30) dashed lines indicate copies measured control experiments lacking SARS-CoV-2 RNA.



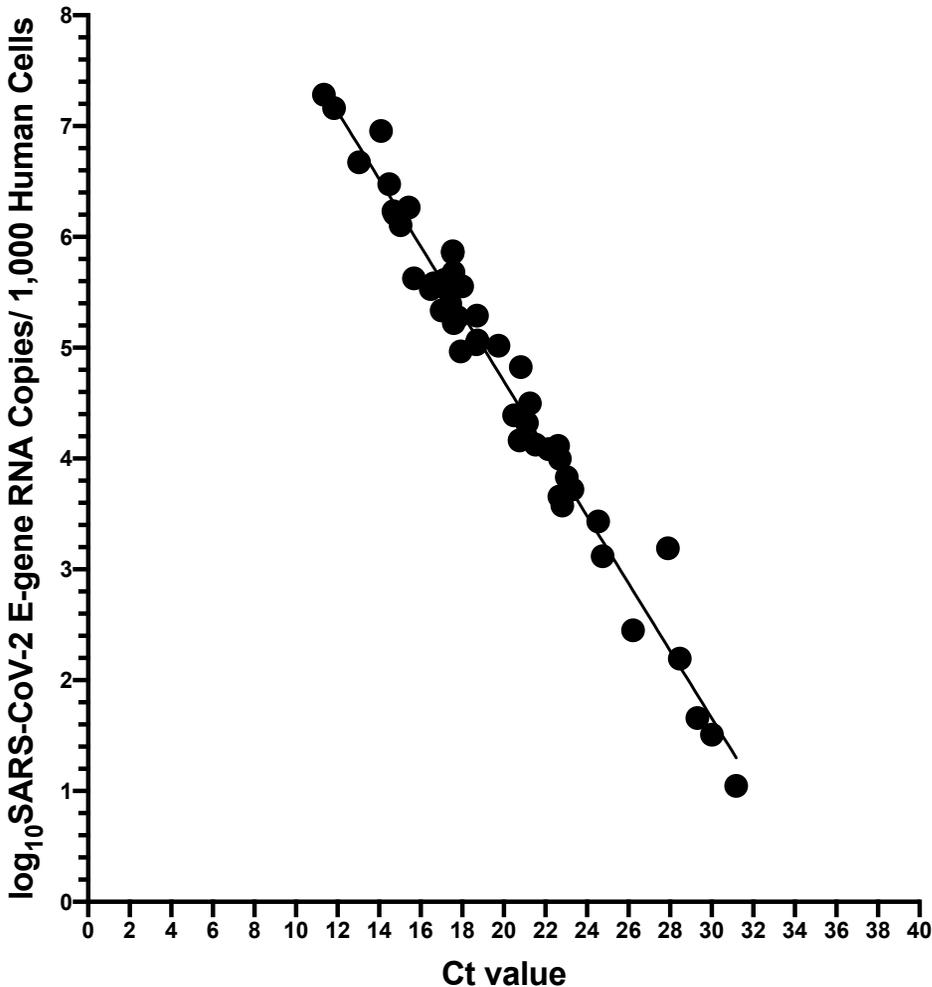
Supplementary Figure 2: Duplexing the IP2 and IP4 primer/probe sets reduces analytical efficiency and precision. (A). Analytical efficiency of SARS-CoV-2 quantification was evaluated for the IP2 and IP4 primer/probe sets when used in separate reactions (dark red and dark blue, respectively) and when duplexed (light red and light blue, respectively), in reactions containing 1,000 and 100 viral RNA input copies. Error bars represent 95% Total Poisson Confidence Intervals. (B). Same as A, but for assay precision (coefficient of variation, CV%).

A**B**

Supplementary Figure 3: Log₁₀SARS-CoV-2 RNA loads in diagnostic specimens, normalized to human cells sampled. (A) SARS-CoV-2 E (green circles), ORF1a (red squares) and ORF1b (blue triangles) gene copy numbers, expressed as RNA copies/1,000 human cells. Line and bars indicate median and interquartile range, respectively. (B) Correlation between SARS-CoV-2 RNA copies/μl extract and RNA copies/1,000 human cells.



Supplemental Figure 4: Residuals of relationship between SARS-CoV-2 RNA copies equivalent and diagnostic test C_t value. Log_{10} Residuals are calculated as log_{10} Measured SARS-CoV-2 RNA copies equivalent minus log_{10} Calculated SARS-CoV-2 RNA copies equivalent from the regression line shown in Figure 6.



$R^2 = 0.97079$

$\log_{10} \text{SARS-CoV-2 E-gene RNA Copies/1,000 Human Cells} = -0.3041(C_t) + 10.8$

Supplemental Figure 5: Relationship between SARS-CoV-2 RNA copies/1,000 human cells and C_t value. Same data as shown in Figure 6, but where the measured SARS-CoV-2 RNA copies/ μ l extract were normalized to copies/1,000 human cells. The linear regression is shown as a solid black line.