Conflict of Interest Disclosure: none to declare

Contact: nkinloch@sfu.ca

Preprint: https://www.biorxiv.org/content/10.1101/2020.12.21.423898v1

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

Natalie N. Kinloch[1,2], Gordon Ritchie[3,4], Winnie Dong[2], Kyle D. Cobarrubias[2], Hanwei Sudderuddin[2], Tanya Lawson[3], Nancy Matic[3,4], Julio S.G. Montaner[2,5], Victor Leung[3,4,5], Marc G. Romney[3,4], Christopher F. Lowe[3,4], Chanson J. Brumme[2,5*], Zabrina L. Brumme[1,2*]

[1]Faculty of Health Sciences, Simon Fraser University, Burnaby BC; [2]BC Centre for Excellence in HIV/AIDS, Vancouver BC; [3]Division of Medical Microbiology and Virology, St. Paul's Hospital, Vancouver, BC; [4]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC; [5]Department of Medicine, University of British Columbia, Vancouver, BC; *equal contribution

BRITISH COLUMBIA CENTRE *for* EXCELLENCE *in* HIV/AIDS

UBC

CIHR IRSC Canadian Institutes of Health Research / Instituts de recherche en santé du Canada

COVID-19 IMMUNITY TASK FORCE

Genome BritishColumbia

SFU

Providence HEALTH CARE How you want to be treated.

BRITISH COLUMBIA Ministry of Health

BACKGROUND and OBJECTIVE:

Quantitative viral load assays hold potential to advance COVID-19 prevention and control. SARS-CoV-2 viral load tests are however not widely available and molecular diagnostics tests typically employ real-time reverse transcriptase (RT)-PCR assays that yield semi-quantitative (C_t value) results only. Reverse transcriptase droplet digital PCR (RT-ddPCR) offers an attractive platform for SARS-CoV-2 RNA quantification. We evaluate eight SARS-CoV-2-specific primer/probe sets developed for real-time RT-PCR diagnostic assays for use on the RT-ddPCR platform. We also derive the equation relating RT-ddPCR-derived SARS-CoV-2 viral loads and real-time RT-PCR C_t values (LightMix® Modular SARS-CoV E gene assay), allowing conversion of existing COVID-19 diagnostic results to viral loads.

METHODS:

Primer/Probe Sets

- Eight real-time RT-PCR diagnostic test primer/probe sets were tested in RT-ddPCR: Charité-Berlin E-Sarbeco, Pasteur Institute IP2 and IP4, China CDC ORF and N, Hong Kong University (HKU) ORF and N and US CDC N1

RT-ddPCR Assay Evaluation

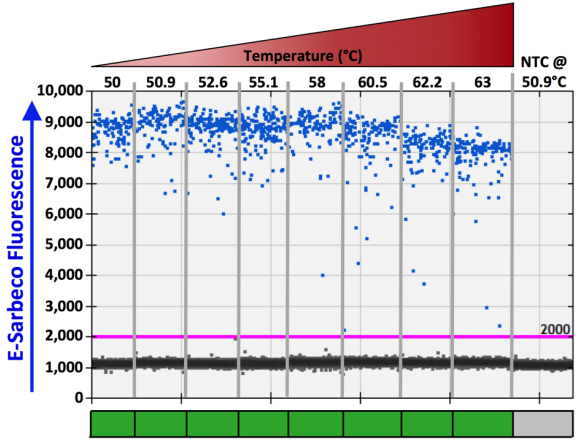
- One-Step RT-ddPCR Kit (BioRad)
- Thermocycling conditions optimized for each set
- Synthetic SARS-CoV-2 RNA standards used in a fixed background of nucleic acids to determine: assay analytical efficiency, precision and, for select primer/probe sets, linear dynamic range and lower limit of detection

Patient Samples

- n= 48 SARS-CoV-2-positive remnant nasopharyngeal swab specimens were re-extracted (BioMerieux EasyMag)
- SARS-CoV-2 RNA was quantified by RT-ddPCR
- real-time RT-PCR diagnostic test C_t values were determined (LightMix® Modular SARS-CoV [COVID-19] E gene assay, implemented on the Roche LightCycler 480)

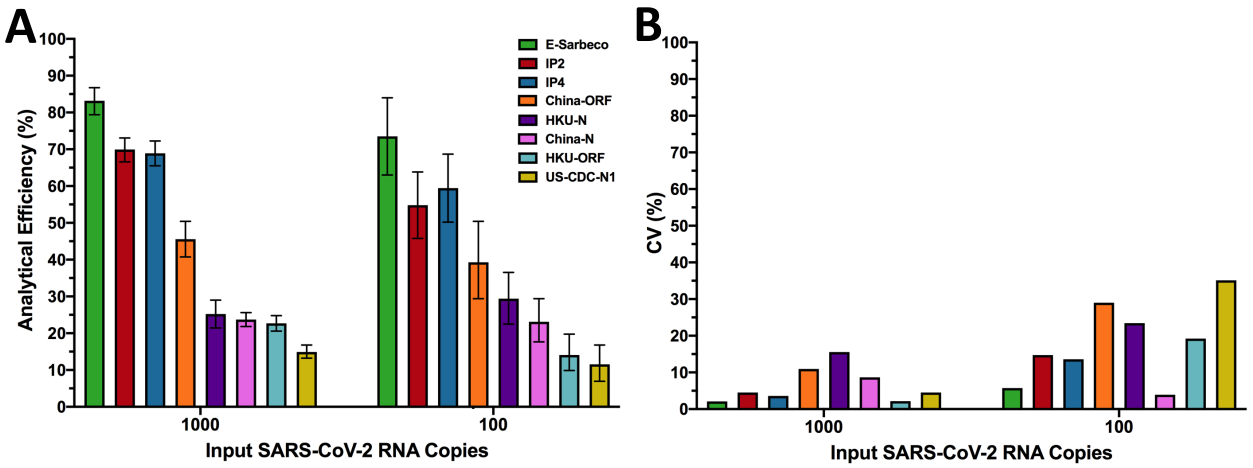
RESULTS (1): RT-ddPCR thermocycling condition optimization

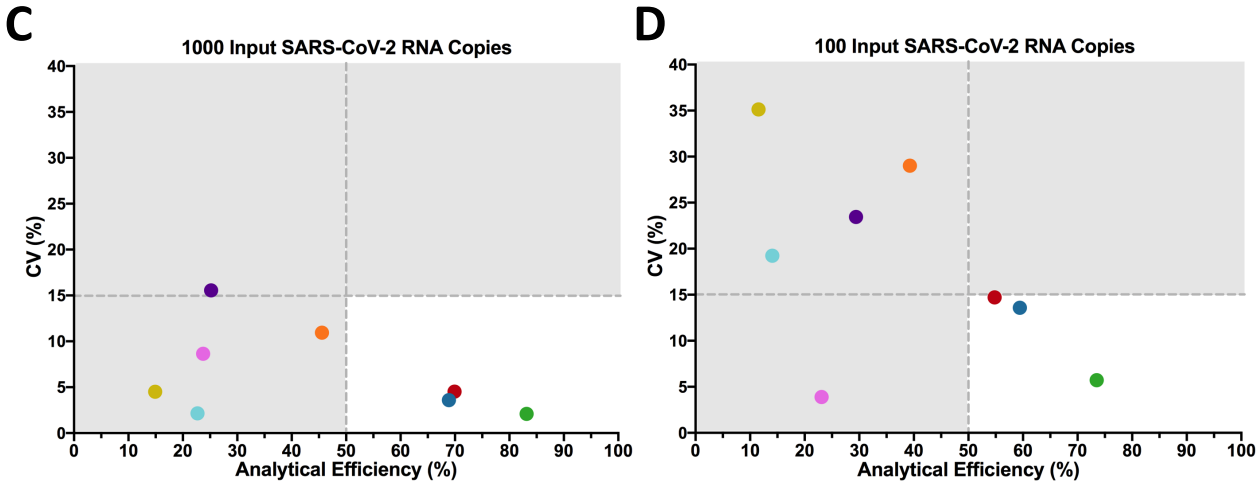
Acceptable Temp. Range (°C)	E-Sarbeco	IP2	IP4	China-ORF	China-N	HKU-ORF	HKU-N	US-CDC-N1
RT	42- 49.7	42- 51.5	42- 50.9	42- 51.5	42.7- 50.9	42- 51.5	42- 51.5	42- 45.7
Annealing/ Extension	50- 63	50- 60.5	50- 60.5	50- 63	50- 60.5	50- 60.5	50.9- 60.5	50- 63



Acceptable temperature ranges modified from the manufacturer's default conditions (reverse transcription [RT]: 42-50°C; annealing/extension: 50-63°C) were determined for each primer/probe set **(Table 1, above)**.

Temperatures that produced insufficient separation of positive from negative droplets, non-specific amplification and/or consecutive 95% confidence intervals of copy number estimates outside those of the maximal point-estimate (not shown) were deemed unacceptable. **Figure 1 (above left)** shows example RT-ddPCR plots for the E-Sarbeco primer/probe set over an annealing/extension step temperature gradient, all test temperatures were acceptable for this primer/probe set.





RESULTS (2): RT-ddPCR assay analytical efficiency and precision

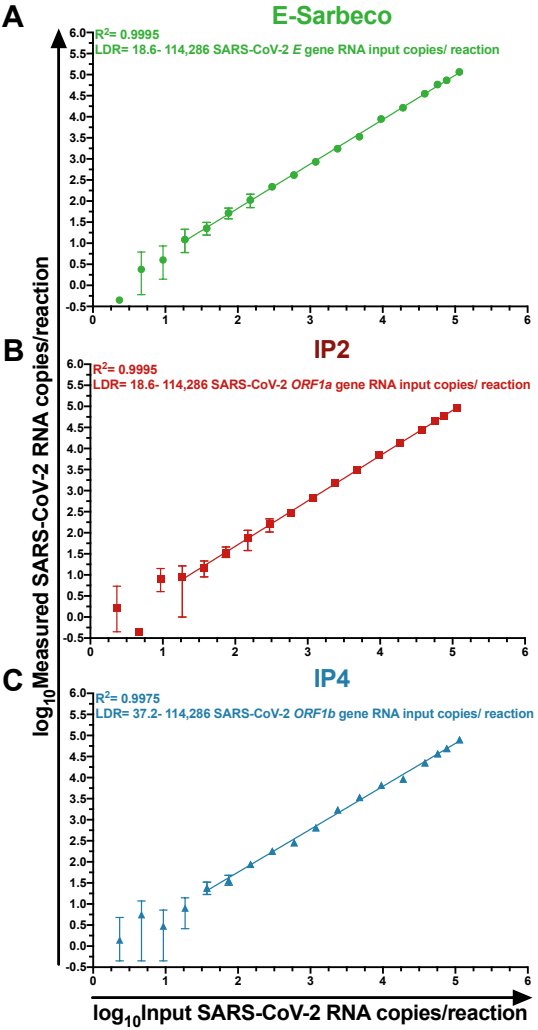
The analytical efficiency (percentage of input viral RNA copies detected by the assay, **Figure 2A**) and precision (coefficient of variation, CV, **Figure 2B**) of SARS-CoV-2 RNA quantification for each primer/probe set was determined at 1000 and 100 SARS-CoV-2 RNA target input copies.

At 1000 input copies, primer/probe set analytical efficiency ranged from 83% (E-Sarbeco) to 15% (US-CDC-N1). At 100 copies, the hierarchy was identical. The E-Sarbeco, IP2 and IP4 sets had the highest analytical efficiencies by a substantial margin. At 1000 target copies, E-Sarbeco analytical efficiency was 83% (95% Total Poisson Confidence Interval [CI]: 79- 87%); IP2, analytical efficiency was 70% (95% CI: 67- 73%); and IP4 analytical efficiency was 69% (95% CI: 66- 72%) (**Figure 2A**). The IP2 and IP4 primer/probe sets were originally designed to be duplexed in real time RT-PCR, but duplexing in RT-ddPCR reduced both efficiency and precision (not shown).

The E-Sarbeco, IP2 and IP4 primer/probe sets were among the most precise when used in RT-ddPCR, with CVs of less than 5% at 1,000 input copies and less than 15% at 100 input copies (**Figure 2B**).

Combined analytical efficiency and precision data confirmed E-Sarbeco, IP2 and IP4 as the best-performing primer/probe sets in RT-ddPCR (**Figures 2C and 2D**), so these were moved forward for further characterization.

Figure 3



RESULTS (3): Linear Dynamic Range (LDR)

The LDR of the E-Sarbeco, IP2 and IP4 assays was determined by iteratively restricting the range of concentrations included in a linear regression of measured versus input SARS-CoV-2 RNA copies (18 two-fold serial dilutions 2.32- 114,286 copies/reaction) to identify the range that maximized the R^2 value and minimized the residuals.

For both E-Sarbeco and IP2, the regression spanned 18.6-114,286 input SARS-CoV-2 RNA copies/reaction, yielding an R^2 value of 0.9995 (**Figure 3A and 3B, left**) and residuals of all included data points were $\pm 0.065 \log_{10}$ copies/reaction (not shown).

The IP4 assay had an estimated LDR of 37.2- 114,286 input copies/ reaction, which yielded an R^2= 0.9975 (**Figure 3C, left**) and residuals of all included data points were $\pm 0.11 \log_{10}$ copies/reaction (not shown).

For all assays, 114,286 input copies/reaction is a conservative estimate of the upper limit of quantification, as saturation of the RT-ddPCR reaction or loss of linearity was still not achieved.

RESULTS (4): Lower Limit of Detection (LLOD)

To determine the LLOD of the E-Sarbeco, IP2 and IP4 RT-ddPCR assays, Probit regression analysis was applied to serial dilutions of synthetic SARS-CoV-2 RNA standards.

The E-Sarbeco RT-ddPCR assay was the most analytically sensitive, with an estimated LLOD of 4.4 (95% Confidence Interval [CI]: 2.4-5.7) SARS-CoV-2 RNA copies/reaction (**Figure 4A, right**).

The estimated LLODs of the IP2 and IP4 assays, respectively, were 7.8 (95% CI: 4.4-10.3) and 12.6 (95% CI: 6.9-16.5) SARS-CoV-2 RNA copies/reaction (**Figure 4B and 4C, right, respectively**).

Figure 4

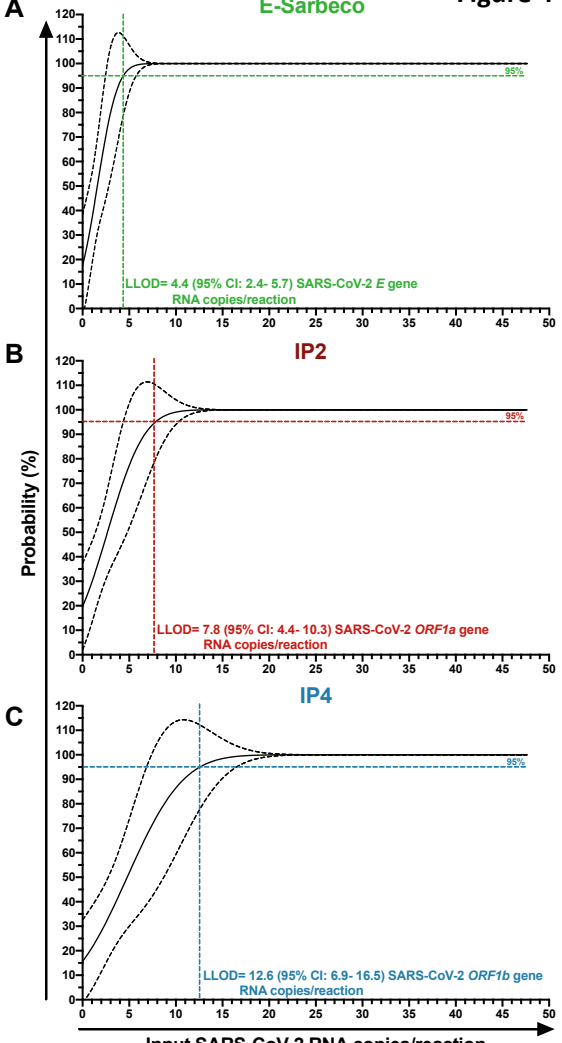


Figure 5

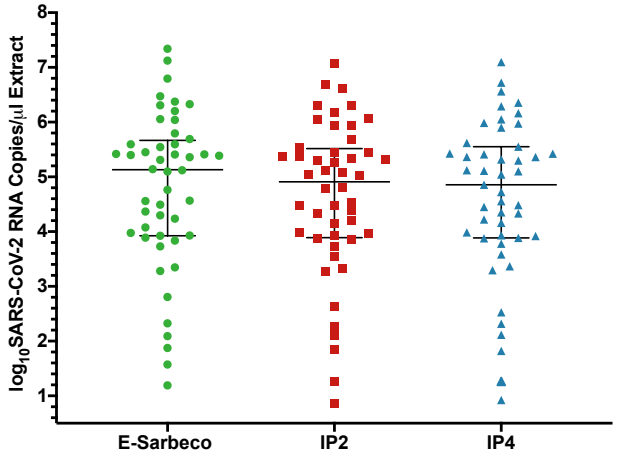
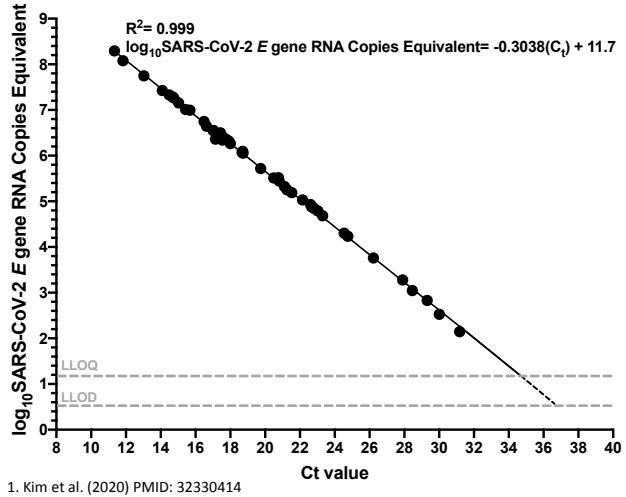


Figure 6



1. Kim et al. (2020) PMID: 32330414

RESULTS (5): SARS-CoV-2 viral load in biological samples

Using the E-Sarbeco, IP2 and IP4 assays, SARS-CoV-2 viral loads were measured in 48 confirmed SARS-CoV-2 positive samples (**Figure 5**).

SARS-CoV-2 RNA in these samples varied over a 6.2 $\log_{10}$ range. Average copy numbers measured using the E-Sarbeco assay (target: *E* gene) were higher than those using the IP2 and IP4 assays (targets: *ORF1a* and *ORF1b*), consistent with assay analytical efficiency and *in vivo* coronavirus RNA expression patterns[1].

The median *E* gene copy number was 5.1 (IQR 3.9- 5.7) $\log_{10}$copies/µl extract compared to medians of 4.9 (IQR 3.9- 5.5) and 4.9 (IQR 3.9- 5.6) $\log_{10}$ copies/µl extract for the IP2 and IP4 targets, respectively. SARS-CoV-2 *E* gene, IP2 and IP4 copy numbers correlated strongly with one another and IP2 and IP4 copy numbers were also highly concordant (Spearman's $\rho>0.99$; $p<0.0001$; Lin's concordance correlation coefficient IP2/IP4, $\rho c=0.9996$ [95% CI: 0.9993- 0.9998]; all not shown).

RESULTS (6): Inferring SARS-CoV-2 viral load from diagnostic C_t value

We characterized the relationship between C_t values produced by a commercial COVID-19 diagnostic platform (LightMix® 2019-nCoV real-time RT-PCR assay, E-gene target, implemented on a LightCycler 480) and SARS-CoV-2 *E* gene RNA copy numbers, normalized to input copies equivalent in the LightMix® assay.

The relationship between C_t value and SARS-CoV-2 RNA copy numbers was log-linear, with an $R^2 = 0.9990$ (**Figure 6**). The relationship between C_t value and absolute SARS-CoV-2 *E* gene copies is given by $\log_{10}$SARS-CoV-2 *E* gene copies equivalent = $-0.3038C_t +11.7$.

A C_t value of 20 for example corresponds to 453,942 (*i.e.* 5.66 $\log_{10}$) SARS-CoV-2 RNA copies. Predicted C_t values corresponding to the E-Sarbeco RT-ddPCR assay LLOQ and LLOD are 34.8 and 36.84, respectively.

SUMMARY AND CONCLUSIONS:

The E-Sarbeco, IP2 and IP4 primer/probe sets, of the 8 originally developed for real-time RT-PCR-based SARS-CoV-2 diagnostic tests evaluated here, were the most efficient, precise and sensitive for RT-ddPCR-based SARS-CoV-2 RNA quantification.

SARS-CoV-2 RNA viral loads in 48 COVID-19-positive diagnostic specimens spanned a 6.2$\log_{10}$ range.

RT-ddPCR-derived SARS-CoV-2 E gene copy numbers calibrated against cycle threshold (C_t) values from a commercial real-time RT-PCR diagnostic platform give a log-linear relationship can be used to mathematically-derive SARS-CoV-2 RNA copy numbers from C_t values, allowing the wealth of available diagnostic test data to be harnessed to address foundational questions in SARS-CoV-2 biology.