



METHOD ARTICLE

SARS-CoV-2 detection by a clinical diagnostic RT-LAMP assay

[version 1; peer review: 1 approved with reservations]

Michael D. Buck ^{1*}, Enzo Z. Poirier ^{1*}, Ana Cardoso^{1*}, Bruno Frederico^{1*}, Johnathan Canton¹, Sam Barrell¹, Rupert Beale¹, Richard Byrne¹, Simon Caidan¹, Margaret Crawford¹, Laura Cubitt ¹, Sonia Gandhi¹⁻³, Robert Goldstone¹, Paul R. Grant⁴, Kiran Gulati⁵, Steve Hindmarsh¹, Michael Howell¹, Michael Hubank^{6,7}, Rachael Instrell ¹, Ming Jiang¹, George Kassiotis ¹, Wei-Ting Lu ¹, James I. MacRae ¹, Iana Martini⁸, Davin Miller⁵, David Moore^{2,3}, Eleni Nastouli^{1,3,9}, Jerome Nicod ¹, Luke Nightingale¹, Jessica Olsen¹, Amin Oomatia⁸, Nicola O'Reilly¹, Anett Rideg¹, Ok-Ryul Song¹, Amy Strange¹, Charles Swanton¹⁻³, Samra Turajlic^{1,6}, Mary Wu¹, Caetano Reis e Sousa ¹,
The Crick COVID-19 Consortium

¹The Francis Crick Institute, London, NW1 1AT, UK²University College London, London, WC1E 6BT, UK³University College London Hospitals NHS Trust, London, NW1 2PG, UK⁴Health Service Laboratories, London, WC1H 9AX, UK⁵New England Biolabs, Ipswich, MA, USA⁶The Royal Marsden Hospital, Sutton, SM2 5NG, UK⁷The Institute of Cancer Research, London, SW7 3RP, UK⁸The Royal Free Hospital, London, NW3 2QG, UK⁹University College London GOS Institute of Child Health, London, WC1N 1EH, UK

* Equal contributors

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Abstract

The ongoing pandemic of SARS-CoV-2 calls for rapid and cost-effective methods to accurately identify infected individuals. The vast majority of patient samples is assessed for viral RNA presence by RT-qPCR. Our biomedical research institute, in collaboration between partner hospitals and an accredited clinical diagnostic laboratory, established a diagnostic testing pipeline that has reported on more than 252,000 RT-qPCR results since its commencement at the beginning of April 2020. However, due to ongoing demand and competition for critical resources, alternative testing strategies were sought. In this work, we present a clinically-validated procedure for high-throughput SARS-CoV-2 detection by RT-LAMP in 25 minutes that is robust, reliable, repeatable, sensitive, specific, and inexpensive.

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report

1. **Michael Knop**, Center for Molecular Biology of Heidelberg University (ZMBH), Heidelberg, Germany

Viet Loan Dao Thi , Heidelberg University

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Hospital, Heidelberg, Germany

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Corresponding authors: Michael D. Buck (michael.buck@crick.ac.uk), Enzo Z. Poirier (enzo.poirier@crick.ac.uk), Caetano Reis e Sousa (caetano@crick.ac.uk)

Author roles: **Buck MD:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Poirier EZ:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Cardoso A:** Formal Analysis, Investigation; **Frederico B:** Formal Analysis, Investigation; **Canton J:** Formal Analysis, Investigation; **Barrell S:** Project Administration; **Beale R:** Conceptualization, Methodology, Project Administration; **Byrne R:** Project Administration; **Caidan S:** Project Administration; **Crawford M:** Project Administration; **Cubitt L:** Project Administration; **Gandhi S:** Conceptualization, Methodology, Project Administration; **Goldstone R:** Software; **Grant PR:** Methodology, Resources; **Gulati K:** Resources; **Hindmarsh S:** Software; **Howell M:** Conceptualization, Methodology, Project Administration, Resources; **Hubank M:** Methodology; **Instrell R:** Resources; **Jiang M:** Resources; **Kassiotis G:** Conceptualization, Methodology, Resources; **Lu WT:** Resources; **MacRae JI:** Conceptualization, Methodology, Project Administration, Resources; **Martini I:** Resources; **Miller D:** Resources; **Moore D:** Conceptualization, Methodology, Software; **Nastouli E:** Conceptualization, Methodology; **Nicod J:** Conceptualization, Methodology, Resources; **Nightingale L:** Software; **Olsen J:** Resources; **Oomatia A:** Resources; **O'Reilly N:** Resources; **Rideg A:** Resources; **Song OR:** Resources; **Strange A:** Resources; **Swanton C:** Conceptualization, Methodology, Project Administration; **Turajlic S:** Resources; **Wu M:** Resources; **Reis e Sousa C:** Conceptualization, Methodology, Project Administration;

Competing interests: D. Miller and K. Gulati are employees of New England Biolabs, which provided the WarmStart Colorimetric LAMP 2X Master Mix used in this work. C. Swanton receives or has received grant support from Pfizer, AstraZeneca, Bristol-Myers Squibb (BMS), Roche-Ventana, Boehringer-Ingelheim, and Ono Pharmaceutical and has consulted for or received an honorarium from Pfizer, Novartis, GlaxoSmithKline, Merck Sharp & Dohme, BMS, Celgene, AstraZeneca, Illumina, Genentech, Roche-Ventana, GRAIL, Medicxi, and the Sarah Cannon Research Institute. C. Swanton also is a shareholder of Apogen Biotechnologies, Epic Bioscience, and GRAIL and has stock options in and is a cofounder of Achilles Therapeutics.

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Introduction

The current pandemic caused by novel coronavirus SARS-CoV-2, first detected in late 2019 in the province of Wuhan, China, has rapidly spread worldwide, infecting more than 68 million individuals as of 11 December 2020¹⁻³. Infection with SARS-CoV-2 can lead to development of COVID-19, a disease associated with severe acute respiratory syndrome, responsible for hundreds of thousands of deaths globally. Controlling the spread of SARS-CoV-2 relies on the ability of healthcare systems to quickly identify infected individuals, which has mainly relied on RT-qPCR for viral RNA detection⁴. International competition for commercial kits and reagents has negatively impacted the ability of many countries to scale up testing capacity to deal with the increased demand caused by rampant infection. Implementing RT-qPCR testing programs requires specialised laboratory equipment and reagents, presenting additional challenges.

In an effort to increase the diagnostic capacity for SARS-CoV-2 infection in the UK, the Francis Crick Institute, a biomedical research institute based in London, rapidly repurposed its staff and facilities in late March 2020 to serve as a clinical diagnostic testing facility through a partnership between a major local healthcare provider (University College London Hospitals National Health Services Trust) and an accredited clinical diagnostic laboratory (Health Services Laboratories, HSL), termed the CRICK COVID-19 Consortium (CCC)⁵. The pipeline utilises a series of in-house buffers to first inactivate patient samples received from care homes and hospitals, and to then extract RNA before using a CE marked commercial kit to detect SARS-CoV-2 by RT-qPCR. Patient results are reported through a custom online web portal that interfaces with the reference laboratory⁵. In order to avoid dependence on any singular testing methodology, to continue increasing testing capacity, and to provide a potential means to deliver diagnostics at the point-of-care, the CCC was also tasked with developing and validating alternative SARS-CoV-2 testing strategies.

Herein, we describe the use of loop mediated isothermal amplification PCR coupled with reverse transcription (RT-LAMP) as a robust method for SARS-CoV-2 detection in clinical specimens⁶. The entire strand displacement and amplification procedure is carried out at a single temperature in less than 30 minutes, alleviating the need for a thermocycler qPCR system. Diagnostic tests utilising this technique have been developed for RNA viruses and other pathogens⁷⁻⁹, and there are now several works focusing on the use of RT-LAMP to detect SARS-CoV-2¹⁰⁻¹⁷. We set up a SARS-CoV-2 RT-LAMP assay using the WarmStart Colorimetric LAMP 2X Mastermix commercialised by New England Biolabs (NEB), which allows for visual assessment of DNA amplification. Alternatively, DNA amplification can be quantified on a real-time PCR machine by complexing the reaction with the DNA dye SYTO 9¹⁰. We made use of primers developed by Zhang *et al.* targeting the nucleocapsid phosphoprotein (N gene) of SARS-CoV-2¹⁰ and, in a parallel reaction, primers that detect human 18S rRNA to control for specimen quality⁹.

Our results demonstrate that within the CCC pipeline, RT-LAMP can readily replace RT-qPCR as a means for detecting SARS-CoV-2 transcripts within RNA extracted from nose-throat swabs and endotracheal secretions/bronchoalveolar lavage fluid. RT-LAMP for the N gene shows absence of non-specific amplification and cross-reactivity with other human coronaviruses or respiratory viruses, and displays a sensitivity threshold almost equivalent to the gold standard RT-qPCR. Switching to RT-LAMP translates into a ten-fold decrease in total reagent cost and a potential four-fold increase in pipeline output. Additionally, we provide preliminary data suggesting that RT-LAMP can be performed without prior RNA extraction, allowing rapid and cost-effective testing that could potentially be extended to point-of-care. The entire workflow was validated under extended governance by public health authorities during the pandemic and inspected by a qualified UKAS assessor against GenQA guidelines to verify compliance to ISO 15189:2012 equivalent standard (US equiv. CAP/CLIA). As such, the procedure developed here is ready to be deployed for diagnostic testing of SARS-CoV-2.

Methods

Patient samples

In response to the pandemic, the Francis Crick Institute formed a partnership with University College London Hospitals (the Crick-COVID-Consortium) and set up a SARS-CoV-2 RT-qPCR testing pipeline (CCC pipeline). Nasopharyngeal swabs tested in this study were obtained from the CCC pipeline⁵.

CCC pipeline procedures and HSL RT-qPCR

All the samples processing through the CCC pipeline was done in accordance with Standard Operating Procedures (SOPs) described recently⁵, which can be found at <https://www.crick.ac.uk/research/covid-19/covid19-consortium>. A redefined reaction, running, and reporting SOP can be found in the Supplemental Methods (*Extended data*¹⁸).

RT-LAMP assay

RT-LAMP reaction was performed in a total volume of 15 μ L, mixing 7.5 μ L WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, M1800), 1.5 μ L of 10X primer mix, 1.5 μ L SYTO 9 Green Fluorescent Nucleic Acid Stain (ThermoFisher Scientific, S34854), 1.5 μ L of Nuclease-free water (Ambion, AM9932) and 3 μ L of sample unless stated otherwise. 10X SYTO 9 solution at 5 μ M in nuclease free water was prepared for a final concentration of 0.5 μ M in the final RT-LAMP reaction. 10X primer mix was prepared from 100 μ M desalted DNA primers obtained from Sigma Custom Oligos Service. 10X primer mixes for N gene and 18S contained FIP and BIP primers at 16 μ M, F3 and B3 at 2 μ M, LF and LB at 4 μ M. RT-LAMP was ran following a SOP (cf. *Extended data*: Supplementary Methods 1 and 2¹⁸) on a 7500, 7500 Fast, QuantStudio 3, 5, or 7 Real-Time PCR System (Applied Biosystems). A negative no template control (NTC) and a positive control (supplied by HSL, SARS-CoV-2 clinical sample) were included on every run. Experiments utilising laboratory grown SARS-CoV-2 were performed in containment level 3 at the Francis Crick

Institute (FCI) by trained personnel according to health and safety guidelines.

We found that the assembled reaction mix was unstable when kept at 4°C for more than a few hours, and sensitive to freeze-thaw when kept at -20°C for more than a day. The RT-LAMP should be performed using freshly prepared reaction mix. The individual components should be stored at -20°C until use and avoid repeated freeze/thaw cycles.

Accreditation and governance

As outlined recently⁵, the CCC was formed in partnership with HSL, a UCLH UKAS accredited lab, who already had a COVID-19 RT-qPCR test in scope. All samples were received and communicated by HSL under their accreditation and the CCC RT-LAMP assay was validated against their existing RT-qPCR test and the CCC's validated RT-qPCR test, which uses a CE marked commercial kit (BGI). Given the urgent timeframe required to implement testing, it was not possible to secure official clinical laboratory accreditation for the FCI. However, full measures were taken to ensure that the CCC RT-LAMP test was evaluated, verified and performed for diagnostic use in an environment that adhered to equivalent international standards (ISO 15189:2012, US equiv. CAP/CLIA), overseen and audited by HSL. These measures were implemented under the advice and oversight of registered professionals from existing nearby ISO accredited medical laboratories, and included writing and following clinical diagnostic SOPs for every stage of the pipeline from sample reception, processing to result reporting by qualified clinical scientists prior to results being communicated to patients by HSL. Additional SOPs were followed for sample storage, disposal of materials, batch certification of reagents and incident reporting. Appropriate risk assessments, training and competency assessment procedures were established and documented. Record sheets were created to document the receipt, batch acceptance testing, and start/end of use dates for key reagents and consumables. An inventory of all key equipment was compiled which, where appropriate, included details of service and calibration records. Systems were also established for the control of all key documents (version implementation, distribution and acknowledgement), audit trailing (what samples were tested when, by whom, with what equipment and using which consumable/reagent batches), and the recording of all untoward incidents/issues (thus facilitating appropriate investigation, rectification and recurrence prevention). Samples were barcoded and tracked using the Crick Clarity library information management system (LIMS). All key documents are available at <https://www.crick.ac.uk/research/covid-19/covid19-consortium>. NHS governance was extended by a specific memorandum of understanding for diagnostic testing between UCLH and the FCI enabled by NHS England. Assurance of the pipeline was performed in collaboration with GenQA, following their checklist for non-accredited laboratories and the lab and CCC workflow were inspected by a qualified UKAS assessor against the GenQA guidelines to verify compliance to ISO15189:2012 equivalent standard.

Results

RT-LAMP test validity

RT-LAMP was performed with 3 µL of RNA in a total reaction volume of 15 µL unless stated otherwise. Each sample was tested in two separate reactions, one with primers targeting the N gene of SARS-CoV-2, and the second targeting human 18S rRNA to control for specimen integrity and quality (Figure 1A). The RT-LAMP mastermix contains a colorimetric pH indicator that turns from pink to yellow upon DNA amplification (Figure 1B). In addition, we benchmarked the RT-LAMP method by measuring DNA amplification using a SYBR based dye in a real-time PCR machine. When measuring fluorescence every minute ('cycle'), double stranded DNA accumulation follows a characteristic exponential amplification phase that eventually plateaus (Figure 1C and 1D).

An initial characterization of the technique was performed using 24 RNA samples purified from patient nasopharyngeal swabs, 12 of which were positive for SARS-CoV-2 as assessed by the CCC pipeline via RT-qPCR⁵. RT-LAMP could detect SARS-CoV-2 in the 12 positive samples with no amplification detected in all 12 negative samples, displaying 100% concordance to our current clinical diagnostic platform (Figures 1C and Figures 1E). Internal control signal was detected in all samples (Figure 1D).

RT-LAMP background noise and specificity

The background signal of the SARS-CoV-2 RT-LAMP assay was assessed by running RNA elution buffer (7 samples) or nuclease free water (7 samples) no template controls (NTCs) performed 4 separate times by 4 different operators. Non-specific signal could be detected in one well of one experiment after 25 minutes (Figure 2A). A similar experiment was performed for the 18S rRNA internal control RT-LAMP assay and revealed signal in NTCs after 20 minutes (Figure 2B). Based on these NTC data, a detection threshold was set for each RT-LAMP assay which eliminated all false positives in the N gene RT-LAMP assay and 21 of 23 false positives in the 18S rRNA RT-LAMP assay (Figure 2C). This translated to a maximum run time for each reaction: 25 minutes for N gene and 20 minutes for 18S (Figure 1C). Implementing these criteria in a separate experiment yielded no false positives from 47 NTCs in the SARS-CoV-2 RT-LAMP protocol and 4.5% false positives in the 18S rRNA RT-LAMP assay (all with a time threshold > 19) (Figure 2D–2E).

We then asked if melting curves obtained from thermocycler runs could be used to help discriminate true positives from false positives. N gene and 18S rRNA RT-LAMP reactions each gave single peaks in melt curve plots (*Extended data: Figure S1A*¹⁸) and consistent melting temperature values (*Extended data: Figure S1B*¹⁸) over multiple experiments performed on positive and negative patient samples. Moreover, positive samples that amplified near the assay endpoint (*i.e.*, high time threshold) still displayed a clear melting curve peak consistent with being true positives (*Extended data: Figure S2A*¹⁸) and were distinguishable from false positives (*Extended data: Figure S2B*¹⁸).

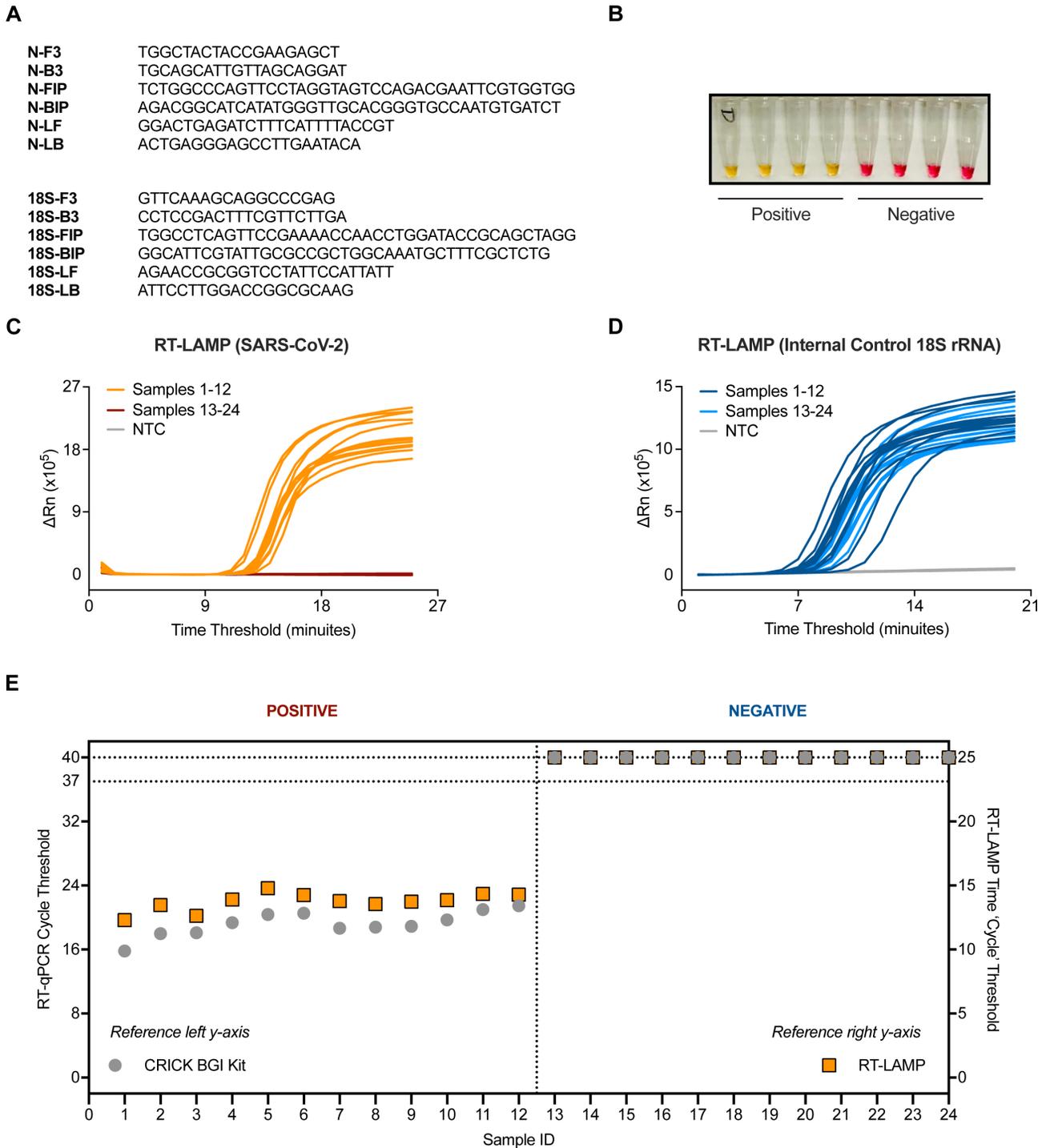


Figure 1. Validation of SARS-CoV-2 detection by RT-LAMP targeting the N gene. (A) The N gene of SARS-CoV-2 was targeted using primers designed by Zhang *et al.*¹⁰, whilst the internal control implemented utilised primers for 18S rRNA initially published by Lamb *et al.*⁹. (B) Upon DNA amplification, a colorimetric pH dye in the reaction mix will change from pink to yellow if the target is present. Four SARS-CoV-2 positive and four SARS-CoV-2 negative patient samples are shown. (C–D) Amplification curves from 24 patient samples assessed by SARS-CoV-2 (N gene) RT-LAMP (C) or 18S (D) in a Real-Time PCR machine. (E) Dot plot of the SARS-CoV-2 Ct values from (C–D) compared to Ct values derived from the Crick’s RT-qPCR diagnostic pipeline using the CE marked BGI kit. Ct values of ‘undetermined’ are plotted as “Ct = 40” (left y-axis) or “Ct = 25” (right y-axis) for illustrative purposes. Data are normalised by assay run/cycles determined by the two methods for comparative purposes. The clinical call from the reference laboratory is indicated above the graph.

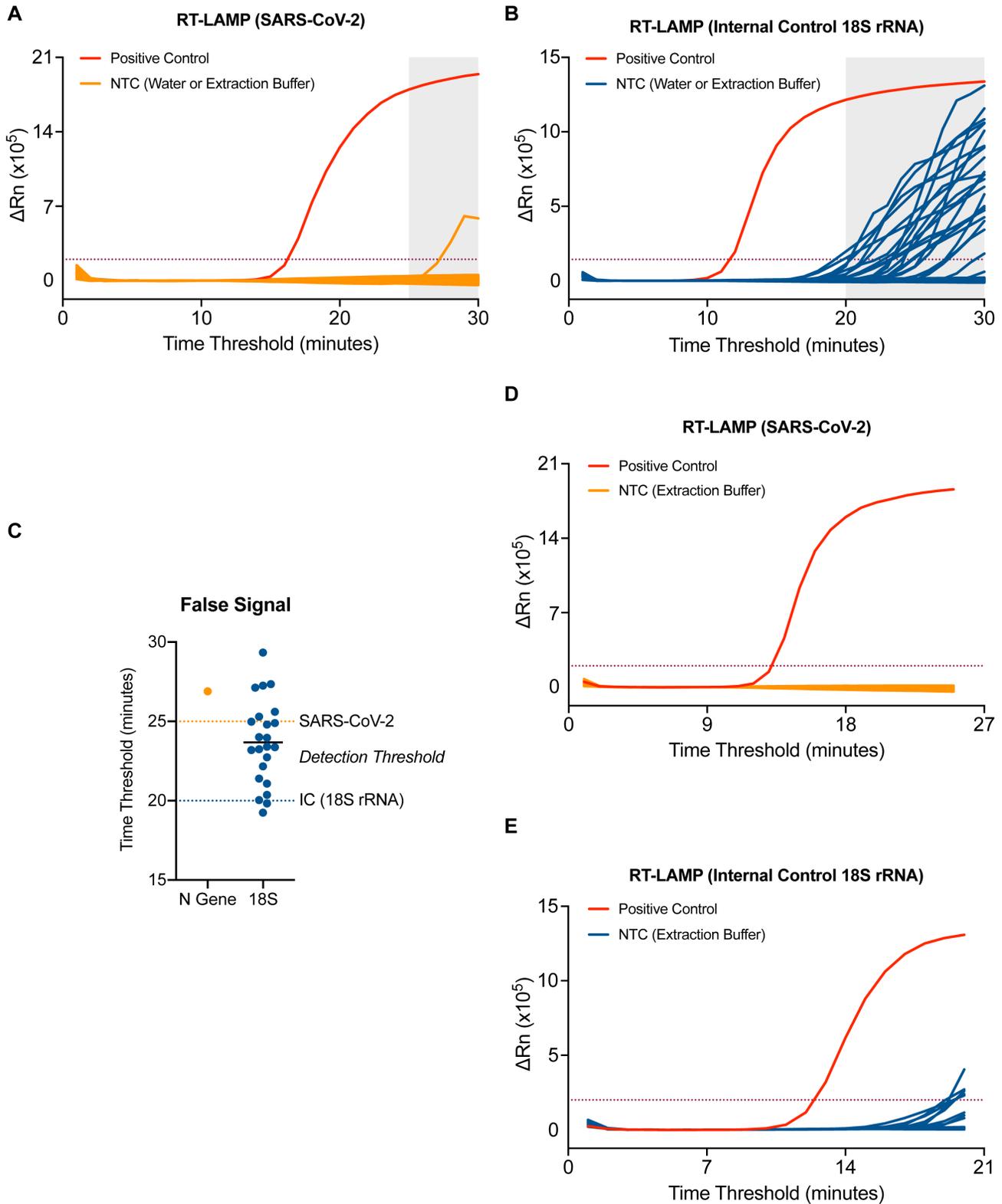


Figure 2. Optimisation of the RT-LAMP assay for accurate detection. (A-B) 7 samples of water and RNA elution buffer from the CCC pipeline were run 4 independent times by 4 distinct operators by SARS-CoV-2. (A) or 18S (B) RT-LAMP. (C) Dot plot of 'Ct' values assessed in (A-B). Assay endpoint detection threshold was set for 18S (dashed blue line) and SARS-CoV-2 (dashed orange line) based on these data. (D-E) RT-LAMP amplification data from 47 samples of RNA elution buffer using the newly established assay endpoint of 25 min for SARS-CoV-2 (D) and 20 minutes for 18S (E).

Therefore, when implementing the assay using a real-time PCR machine (thermocycler) with SYBR-based detection, incorporation of a traditional melting curve stage allows for elimination of false positives and increases reporting confidence for SARS-CoV-2 positive samples (see *Extended data: Supplemental Methods*²¹).

To further assess the specificity of the N gene RT-LAMP assay, we first determined possible cross-reactivity with human RNA. 95 wells of RNA from a human cell line extracted by the CCC pipeline gave no signal in the assay (*Figure 3A*). To assess the assay's specificity for SARS-CoV-2, the RT-LAMP assay was performed on RNA purified from clinical samples

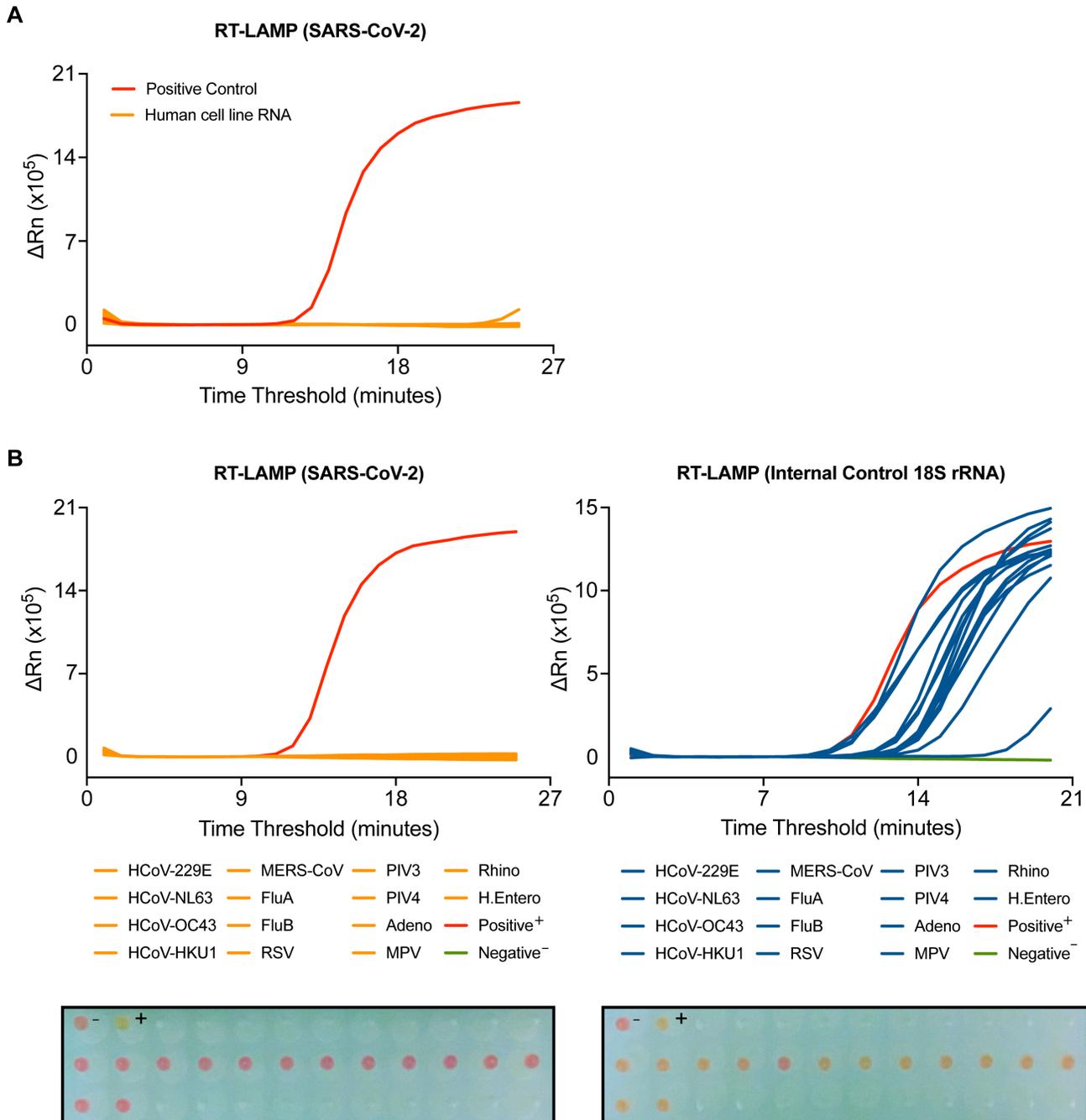


Figure 3. SARS-CoV-2 RT-LAMP is highly specific. (A) SARS-CoV-2 RT-LAMP was performed on 95 wells of human cell line RNA extracted by the CCC pipeline. (B–C) RT-LAMP targeting SARS-CoV-2 (B) or 18S (C) was performed on COVID-19 negative patient samples with positively identified with other viral infections, including human coronaviruses (HCoV), influenzas (Flu), respiratory syncytial virus (RSV), parainfluenzavirus (PIV), adenovirus (Adeno), metapneumovirus (MPV), rhinovirus (Rhino), and human enterovirus (H. Entero). The colorimetric read-out from the actual run is depicted below. NTC (-) and positive control (+).

from COVID-19 negative patients infected with a variety of RNA viruses, including seasonal coronaviruses strains 229E, NL63, OC43 and HKU1, MERS-CoV, Influenza A and B viruses, metapneumovirus (MPV), respiratory syncytial virus (RSV) or parainfluenza viruses type 3 and 4 (PIV3, PIV4). All samples gave a signal in the 18S rRNA RT-LAMP, confirming the presence of human RNA (Figure 3B). With the N gene RT-LAMP, no signal was observed with any of the 14 distinct virus containing specimens tested, confirming that the assay is highly specific for SARS-CoV-2 (Figure 3B).

RT-LAMP sensitivity and precision

In order to ascertain the sensitivity of the N gene RT-LAMP assay, RNA from a SARS-CoV-2 quantified gene copy number standard obtained from the UK National Institute for Biological Standards and Control (NIBSC) was extracted and assessed by limiting dilution. The results indicate that the limit of reliable detection of the N gene RT-LAMP assay is between 500–1000 copies of N gene (Figure 4A and 4B) introduced in the assay. As the reaction is performed with 4.5 μL of sample, this assay presents a limit of detection of 1.1 - 2.2 × 10⁵ N gene copies

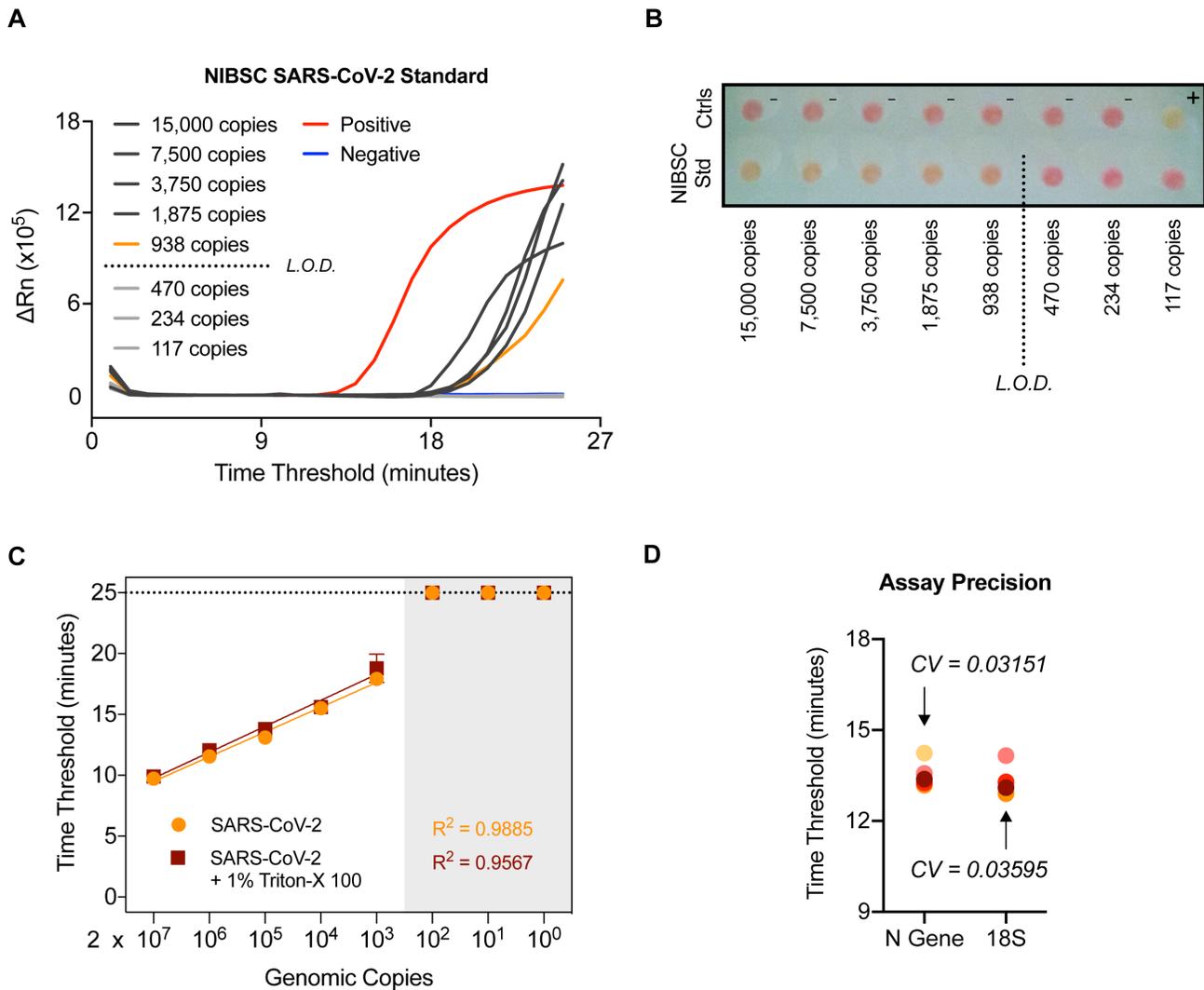


Figure 4. SARS-CoV-2 RT-LAMP is highly sensitive, robust, and precise. (A–B) NIBSC SARS-CoV-2 standard was serially diluted and the indicated number of copies in 4.5 μL was assessed by N gene RT-LAMP. Amplification curves shown with the limit of detection (L.O.D.) determined by the presence or absence of amplification following the depicted dilution in (A) or via colorimetric read-out (B). (C) RNA extracted from laboratory grown SARS-CoV-2 was serially diluted 10-fold and assessed by RT-LAMP in the presence or absence of 1% Triton-X 100. (D) A COVID-19 positive patient sample was RNA extracted independently 5 times through the CCC pipeline and subjected to 5 independent N gene RT-LAMP reactions. Assay precision for N gene and 18S was determined by calculating the coefficient of variation between Ct values observed (CV).

per mL. This was confirmed using 10-fold serial dilutions of RNA extracted from laboratory-grown SARS-CoV-2 quantified using the NIBSC standard (Figure 4C). Notably, a highly linear response was observed, even in the presence of 1% Triton-X 100, which has been proposed as a means of inactivating SARS-CoV-2¹⁹.

The RT-LAMP assay reproducibility and precision were determined by extracting RNA 5 times from a confirmed COVID-19 positive patient sample through the CCC pipeline and assessing by N gene and 18S RT-LAMP in 5 independent experiments, performed by two different operators (Figure 4D). The coefficient of variation was 0.03151 for the N gene RT-LAMP analysis and 0.03595 for the 18S rRNA internal control. We also verified that RT-LAMP can be performed in a 384-well plate format using a QuantStudio 5 real-time PCR machine with equivalent results (Extended data: Figure S3¹⁸).

Clinical validation of RT-LAMP against two clinical diagnostic RT-qPCR assays

The RT-LAMP assay was benchmarked against the RT-qPCR methods used by the reference clinical diagnostic laboratory, HSL, and that used by the CCC validated clinical diagnostic platform by assessing 37 clinical samples processed in parallel by both laboratories with duplicate RT-qPCR analyses. RNA extracted by the CCC pipeline was then tested by RT-LAMP in duplicate runs. RT-LAMP detected 16 positives in both experiments, which was 100% concordant with results obtained by the CCC's RT-qPCR assay using a CE marked kit from BGI and HSL's N gene RT-qPCR assay. However, positives with Ct > 35 near the limit of detection (Ct = 37) of the BGI RT-qPCR assay, which were termed 'borderline positives', were not consistently detected by the N gene RT-LAMP assay (Figure 5A). In an additional set of experiments, 71 clinical specimens in viral transport medium (VTM) stored at HSL were subjected to RNA extraction through the CCC pipeline and examined by HSL's RT-qPCR and CCC's RT-LAMP assay in duplicate (Figure 5B). Again, samples with Ct < 39 in the HSL RT-qPCR analysis (which runs for 45 cycles as opposed to 40 in the BGI assay) were confidently detected by RT-LAMP, whilst samples with Ct values near the limit of detection (39 < Ct < 41), indicating very low levels of SARS-CoV-2 RNA, displayed inconsistent amplification by N gene RT-LAMP. Altogether, these data demonstrate that RT-LAMP performed on extracted RNA accurately detects SARS-CoV-2 in clinical samples as verified by a clinical diagnostic laboratory and a validated clinical diagnostic pipeline. However, samples with Ct values within two cycles of the limit of detection of either RT-qPCR assays, were not consistently identified in duplicate RT-LAMP runs, with detection for some samples in one run, but not always in the other. These results suggest that RT-LAMP is slightly less sensitive than RT-qPCR when performed with extracted RNA. However, it is important to bear in mind that the RT-LAMP assay was performed during these validation experiments with a third of the input RNA used by either RT-qPCR methods during

these validation experiments (3 µL versus 10 µL), partly due to limitations in sample availability.

Discussion

Rapid and reliable detection of SARS-CoV-2 is required to efficiently diagnose infected individuals and to provide governments and health systems with guidance for treatment and quarantine strategies to reduce the risk of transmission. The CCC was formed to address a critical testing shortage in the London area, especially for frontline healthcare workers, with the additional goal of rapidly validating and disseminating SOPs for others to scale up their own diagnostic programs. The majority of testing is currently performed by RT-qPCR amplification of viral RNA obtained from nasopharyngeal samples. In the face of continued global demand and competition for reagents and resources and to minimise reliance on any singular testing strategy, we have outlined here procedures to utilise RT-LAMP as a cost-effective and high throughput alternative to RT-qPCR for detecting SARS-CoV-2 in clinical specimens.

Implementing this testing method would reduce the CCC's operational costs ten-fold when accounting for all consumables (data not shown), provide the ability to scale up our output further (Extended data: Figure S3¹⁸), and decrease reporting turnaround time in comparison to our current method. We confirmed that the assay is highly specific for SARS-CoV-2 and displays lack of cross-reactivity with other respiratory viruses, including seasonal coronaviruses. Assay accuracy and robustness is denoted by an absence of false positives and the further introduction of a melt curve stage allows increased confidence in the identification and reporting of genuine positives. When performed with extracted RNA, the N gene RT-LAMP assay displays a sensitivity comparable to clinically approved RT-qPCR methods. Additionally, preliminary experiments demonstrate that RT-LAMP can be performed directly without RNA extraction, by inactivating virus-containing dry swabs in a detergent-based solution. However, these experiments did not make use of true nasopharyngeal swabs that are likely to contain material that can cause RNA degradation or inhibit the assay (unpublished observations).

Our methodology makes use of a real-time qPCR machine that measures DNA amplification using a SYBR based dye, allowing for real-time detection and standardised reporting. We did not test extensively if RT-LAMP could be assessed by the colorimetric indicator alone, although some of our data suggests that the colour change readout is concordant with the SYTO 9 dye results (Figure 1, Figure 3 and Figure 4). If RT-LAMP were coupled with a colorimetric read-out and the need for RNA extraction could be obviated, the result would be a testing modality that tremendously reduces the cost and time for SARS-CoV-2 diagnostics and allow its application at point-of-care and in remote areas where sophisticated testing infrastructures currently do not exist.

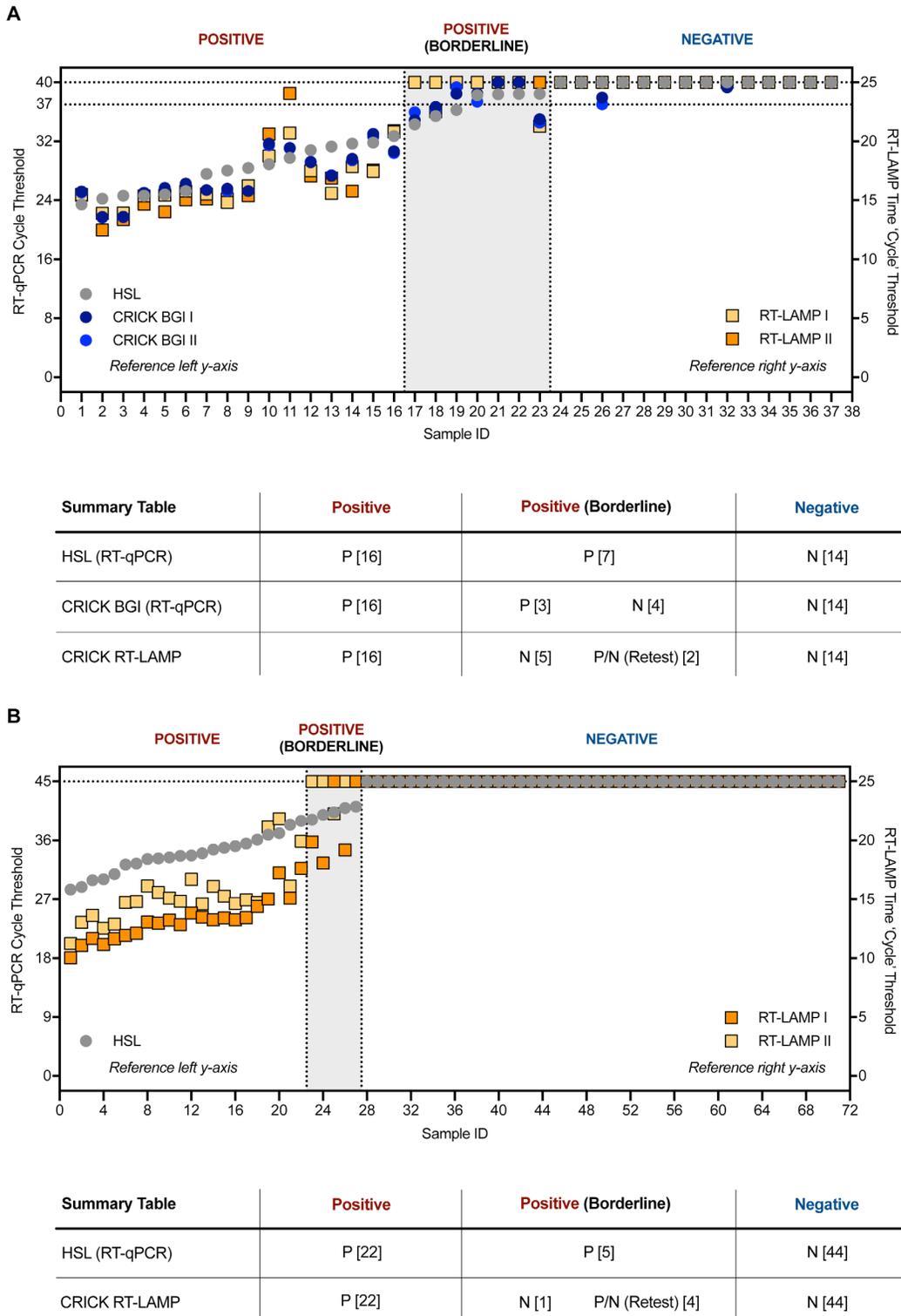


Figure 5. Clinical validation of SARS-CoV-2 RT-LAMP. (A) 37 patient samples were processed in parallel by HSL and the CCC pipeline and interrogated by HSL's RT-qPCR (N gene) and the CCC's BGI RT-qPCR (ORF1a) in duplicate. RNA left from the CCC pipeline was assessed in two separate experiments by N gene RT-LAMP. The graph indicates Ct values for HSL and CCC RT-qPCR runs by left y-axis and 'Ct' time thresholds for RT-LAMP via the right y-axis. Data are normalised by assay run/cycles determined by the two methods for comparative purposes. The clinical call from the reference laboratory is depicted above the summary table provided below. Positives (P) with low Ct values were reliably detected by RT-LAMP, whilst 'borderline positive' (samples with Ct values near the limit of detection of each RT-qPCR assay) were inconsistently detected, displaying no visible amplification at times – negative (N). (B) 71 VTM samples from HSL were RNA-extracted through the CCC pipeline and interrogated by HSL RT-qPCR (45 cycles), or by two independent N gene RT-LAMP experiments (25 minutes

Data availability

Underlying data

Open Science Framework: SARS-CoV-2 detection by a clinical diagnostic RT-LAMP assay; raw data for figures, <https://doi.org/10.17605/OSF.IO/2XR8U>²⁰.

Open Science Framework: SARS-CoV-2 detection by a clinical diagnostic RT-LAMP assay; raw data for supplementary figures, <https://doi.org/10.17605/OSF.IO/YFCSW>²¹.

Extended data

Open Science Framework: SARS-CoV-2 detection by a clinical diagnostic RT-LAMP assay, supplementary figures and methods, <https://doi.org/10.17605/OSF.IO/PAF36>¹⁸.

This project contains the following extended data:

- **Figure S1. SARS-CoV-2 RT-LAMP and internal control 18S rRNA RT-LAMP display a consistent dissociation peak and melting temperature.** (A) Dissociation curves are shown for 11 SARS-CoV-2 positive and 11 negative clinical samples along with 12 NTC wells tested by RT-LAMP N gene assay (top) and 18S assay (bottom). (B) Dot plot of the melting temperatures determined by 94 positive patient samples and 235 negative patient samples tested by RT-LAMP assay. Data are pooled from six separate experiments performed on 3 separate RT-PCR machines. The average melting temperature (T_m) is depicted by a dotted line in (A) and below the graph in (B) with the standard of deviation.
- **Figure S2. Inclusion of a melt curve stage complements the ability of RT-LAMP to accurately distinguish true SARS-CoV-2 positive samples.** (A) Amplification curves (top) and dissociation curves (bottom) are depicted from a range of positive samples for each assay along with a negative sample for reference. TP indicates a true positive call based on amplification above background plus consistent melting curve and T_m consistent with genuine positives. (B) Amplification curve (left) and dissociation curve (right) are depicted of positive samples and NTCs. TP indicates the same criteria outlined in (A), whereas FP denotes a false positive, which despite amplifying above background, does not have a melting curve and temperature consistent with genuine positives.
- **Figure S3. RT-LAMP yields identical results across different RT-PCR instruments and 96 versus 384-well plate formats.** Dot plot comparing results from RT-LAMP assays (N gene, top; 18S, bottom) on the same clinical positive and negative sample serially diluted 2-fold using an ABI 7500 Fast or QuantStudio 5 machine (96-well vs. 384-well plate format).
- Supplementary Method 1: RT-LAMP Mastermix Preparation
- Supplementary Method 2: RT-LAMP RNA Loading and Running RT-PCR Machine
- Supplementary Method 3: RT-LAMP Results Reporting

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](https://creativecommons.org/licenses/by/4.0/) (CC0 1.0 Public domain dedication).

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Jerome Nicod¹, Luke Nightingale¹, Stephanie Nofal¹, Paul Nurse¹, Savita Nutan², Caroline Oedekoven¹, Anne O'Garra¹, Jean D. O'Leary¹, Jessica Olsen¹, Olga O'Neill¹, Nicola O'Reilly¹, Paula Ordonez Suarez¹, Neil Osborne¹, Amar Pabari⁷, Aleksandra Pajak¹, Venizelos Papayannopoulos¹, Namita Patel¹, Yogen Patel⁵, Oana Paun¹, Nigel Peat¹, Laura Peces-Barba Castano¹, Ana Perez Caballero², Jimena Perez-Lloret¹, Magali S. Perrault¹, Abigail Perrin¹, Roy Poh⁵, Enzo Z. Poirier¹, James M. Polke⁵, Marc Pollitt¹, Lucia Prieto-Godino¹, Alize Proust¹, Clinda Puvirajasinghe², Christophe Queval¹, Vijaya Ramachandran², Abhinay Ramaprasad¹, Peter Ratcliffe¹, Laura Reed², Caetano Reis e Sousa¹, Kayleigh Richardson¹, Sophie Ridewood¹, Fiona Roberts¹, Rowenna Roberts², Angela Rodgers¹, Pablo Romero Clavijo¹, Annachiara Rosa¹, Alice Rossi¹, Chloe Roustan¹, Andrew Rowan¹, Erik Sahai¹, Aaron Sait¹, Katarzyna Sala¹, Theo Sanderson¹, Pierre Santucci¹, Fatima Sardar¹, Adam Sateriale¹, Jill A. Saunders¹, Chelsea Sawyer¹, Anja Schlott¹, Edina Schweighoffer¹, Sandra Segura-Bayona¹, Rajvee Shah Punatar¹, Joe Shaw², Gee Yen Shin^{6,7}, Mariana Silva Dos Santos¹, Margaux Silvestre¹, Matthew Singer¹, Daniel M. Snell¹, Ok-Ryul Song¹, Moira J. Spyer³, Louisa Steel², Amy Strange¹, Adrienne E. Sullivan¹, Charles Swanton^{1,3,6}, Michele S.Y. Tan¹, Zoe H. Tautz-Davis¹, Effie Taylor¹, Gunes Taylor¹, Harriet B. Taylor¹, Alison Taylor-Beadling², Fernanda Teixeira Subtil¹, Berta Terré Torras¹, Patrick Toolan-Kerr^{1,3}, Francesca Torelli¹, Tea Toteva¹, Moritz Treeck¹, Hadija Trojer^{1,3}, Ming-Han C. Tsai¹, James M.A. Turner¹, Melanie Turner⁷, Jernej Ule^{1,3}, Rachel Ulferts¹, Sharon P. Vanloo^{1,3}, Selvaraju Veeriah³, Subramanian Venkatesan¹, Karen Vousden¹, Andreas Wack¹, Claire Walder², Philip A. Walker¹, Yiran Wang¹, Sophia Ward^{1,3}, Catharina Wenman², Luke Williams¹, Matthew J. Williams¹, Wai Keong Wong⁶, Joshua Wright¹, Mary Wu¹, Lauren Wynne¹, Zheng Xiang¹, Melvyn Yap¹, Julian A. Zagalak^{1,3}, Davide Zecchin^{1,11} and Rachel Zillwood¹

¹The Francis Crick Institute, London NW1 1AT, UK

²Great Ormond Street Hospital for Sick Children NHS Foundation Trust, London WC1N 3JH, UK.

³University College London, London WC1E 6BT, UK

⁴London School of Hygiene & Tropical Medicine, London WC1E 7HT, UK

⁵National Hospital for Neurology and Neurosurgery, University College London Hospitals,

NHS Foundation Trust, London WC1N 3BG, UK

⁶University College London Hospitals, NHS Foundation Trust, London NW1 2PG, UK

⁷Health Services Laboratories, London WC1H 9AX, UK

⁸The Institute of Cancer Research, London SW7 3RP, UK

⁹The Royal Marsden Hospital, Surrey SM2 5NG, UK

¹⁰Public Health Wales, Heath Park, Cardiff CF14 4XW, UK

¹¹University College London GOS Institute of Child Health, London WC1N 1EH, UK

¹²Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, London NW1 0TU, UK

¹³Royal Free London NHS Foundation Trust, London NW3 2QG, UK

CAPTURE study: Nicky Browne¹, Kim Edmonds¹, Yasir Khan¹, Sarah Sarker¹, Ben Shum¹, Tim Slattery¹, & Samra Turajlic^{1,2}

¹The Royal Marsden Hospital, Surrey, SM2 5NG, UK

²The Francis Crick Institute, London NW1 1AT, UK

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Michael Knop

Center for Molecular Biology of Heidelberg University (ZMBH), Heidelberg, Germany

Viet Loan Dao Thi

Center of Infectious Diseases, Virology, Heidelberg University Hospital, Heidelberg, Germany

Summary:

In the ongoing pandemic, alternative methods to detect SARS-CoV-2 infections at a scalable, faster, and cheaper level, are still in need. In this manuscript, Buck *et al.* report on the use of the NEB warmstart enzyme preparation for colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) in combination with primers published by NEB (Zhang *et al.*) for the amplification and detection of SARS-CoV-2 viral RNA in clinical samples. For detection, a colorimetric implementation of the RT-LAMP assay (based on a pH shift and color change of an indicator dye upon amplification of nucleic acids) along with a fluorescent dye for DNA detection was used. The assay was tested for COVID-19 diagnostics and the report includes data on sensitivity, specificity as well as a positive sample control reaction. All established procedures were set up in agreement with the requirements for patient diagnostics, including SOPs for all relevant procedures. The authors furthermore claim that the cost of the assays would be a fraction of the ones used for RT-qPCR.

Major points:

The authors use colorimetric LAMP reagents but then add a green fluorescent dye for the detection of the fluorescence signal using a light cycler throughout their experiments, with the exception of the few figure panels where they show color images of the reaction tubes? Is this correct?

Importantly, the authors advertise the speed of the RT-LAMP assay in the abstract (25 min). This is somewhat misleading to non-expert readers who might think that the total time of the procedure (from sample taking to result) takes only that long. But the time to isolate the RNA and to set up the assays also needs to be considered.

The preprint in its current form appeared online on July 2020, and the authors claim clinical validation of the assay, higher speed, and almost similar sensitivity to qPCR at much lower costs. Given these advantages, may I ask whether the assay has been used for SARS-CoV-2 diagnostics? If yes, for how many samples? It would be essential to present such data including statistics from validation of LAMP positives by qPCR. On the other hand, if the assay was not used for diagnostics, it would be good to carefully explain the reasons behind it; otherwise, this may raise concerns about the validity of some of the data and acclaimed features of the assay.

Average RT-qPCR assays using well-established primer sets meanwhile reach sensitivities where 10 molecules of the viral genome are detected in 95% of the cases, which for most RT-qPCR kits corresponds to a CT approx. = 37. This can be considered as standard. Is this given for the used PCR assay (CRICK BGI)?

The manuscript does not elaborate on the used RNA isolation procedures. This should be included in the methods section. Different RNA extraction protocols exist, either using silica matrix columns or magnetic beads. These protocols allow purification and simultaneous concentration of nucleic acids relative to the original specimen. For diagnostic analysis of nucleic acids in SWAB specimen, a concentration factor of 2 to 5 usually applies. This yields a LOD of approx. 500 or fewer viral RNA molecules per ml of patient specimen for an RT-qPCR that uses 10 μ l input volume. The authors here claim a sensitivity of the RT-LAMP of 11000-22000 molecules per ml patient specimen using a SARS-CoV-2 standard (Figure 4), while they claim a sensitivity up to Ct=35 with patient samples (Figure 5a). A Ct=35 in an RT-qPCR assay corresponds roughly to 40 molecules per 10 μ l purified RNA, while only 3 μ l are used for the RT-LAMP, which would imply a sensitivity limit of approx. 15 molecules per reaction. This is inconsistent with the data shown in Figure 4 and with previous reports for the N-gene LAMP primer sets used here, which was reported to have a sensitivity in the range of 100 molecules per reaction (e.g. ¹). A closer inspection of the data shown in Figure 5a indicates that all RT-LAMP positive samples have Ct<<32 (Figure 4a), but not enough samples in the Ct range of 25 – 35 are shown to confidently estimate a Ct value that corresponds to the LOD. These issues should be clarified and, if necessary, faulty standard calibrations or additional means to calibrate the sensitivity should be used.

The authors represent a consortium that has generated more than 252000 RT-qPCR results since the beginning of the pandemic. Obviously, knowing the exact sensitivity limit of the RT-LAMP assay and the data of the RT-qPCR would permit an estimation of the false-negative rate of the RT-LAMP assay in a much more realistic scenario as compared to the limited number of samples presented here.

The assay makes use of one primer set only for SARS-CoV-2 detection. Meanwhile, many more primer sets have been described, and a publication by NEB scientists ² furthermore presented means to improve the sensitivity of the assay by adding GuHCL to the reaction buffer and by using multiple primer sets in combination. This could be discussed and the benefits of using better primers/conditions to be estimated.

Specificity: The authors elude that the RT-LAMP assay does not cross-react with other viruses. If the authors really want to use this experiment as a valid assay to demonstrate the sensitivity of the RT-LAMP assay for SARS-CoV-2, they should provide evidence that the used samples indeed contain viral nucleic acids in sufficient numbers (> 10000 molecules per ml) of the patient

specimen. Another issue that needs to be addressed is the relatively well-established propensity of RT-LAMP to produce false-positive results by spurious amplification (e.g. see ³). The frequency of these may be small, i.e. below 1%, and hence such cases escaped detection in this work. As shown by the authors, melting curves allow to identify those, but upscaling and the use of the colorimetric RT-LAMP will certainly bear the risk of false positives. Here, validation of positives by RT-qPCR could be used.

Additional points:

The authors mention twice preliminary data on an RNA-extraction free procedure. Where are these data? I suggest either adding the data or removing the corresponding sentences in the introduction and discussion.

The number of cells in a swab can be affected by many factors. Even in the absence of human cells/detectable housekeeping genomes, viral RNA can be or should be present in a positive sample in the form of cell-free, viral particles. How do the authors suggest proceeding if 18S rRNA is not detected in a sample? How does the detection of the housekeeping gene affect the analysis of the clinical samples (e.g. in Figure 5)?

For example, in Figure 3B. The fifth sample appears to be 18S rRNA negative, as the reaction is still pink. How is such a result taken into account?

The citations are not exhaustive. Many manuscripts have been published meanwhile that make use of the colorimetric RT-LAMP assay for COVID-19 diagnostics. In addition, a description of the major differences or similarities to previously published work is missing.

The manuscript would benefit from a report on the frequency of mutations in the binding sequences of the primer set used, which could lead to false negatives. This can easily be done using online resources (<https://www.gisaid.org> or <https://covidcg.org>) and the great number of viral genomes sequenced in the UK.

The authors need to report on measures to avoid contamination by LAMP products. This is an important point that may be less relevant for a diagnostic setting (since people are aware of this issue), but in an academic environment, researchers may need a reminder, which could be given for example by pointing out relevant literature ⁴ or by indicating strategies to eliminate those in the first place (UNG/UDG), or both.

Specific comments to the Figures:

Figure 1: Suggest moving primer sequences to a separate table.

Figure 2: Panels D and E are redundant with Panels A and B as the threshold is indicated by the grey background (just add an explanation of the threshold to the legend). Panel C is also redundant and difficult to read, the information is already in A and B. Give an explanation of the false negatives on the 18S control, which already give fluorescent signals at 20 mins into the LAMP reaction. The primer design seems suboptimal as spurious amplification usually occurs at later time points. At what time point did the authors analyze the colorimetric read-out for 18S, e.g. in Fig 3B. Please define up to when is the reaction reliable.

Suggest moving the melting curves from S1A to the main figures. When calculating sensitivity thresholds-does the melting curve change anything? Does it improve the sensitivity?

Figure 3B, right panel: All different samples are just blue lines. Like this, it is impossible for the reader to understand which line corresponds to which sample. As the coloration is somewhat different between the samples as well as the time points where the fluorescent signal becomes positive, it would be good to see if some sort of correlation exists or not.

Figure 4 C: I do not understand the reason behind treating already purified SARS-CoV-2 RNA with Triton X. This only makes sense in an extraction-free setting.

Figure 4 D: When determining the RT-LAMP assay precision, why extracting the RNA 5 times independently and then running the assay? I expect higher variations from the RNA extraction than from the RT-LAMP assay itself, which should be highly stable and reproducible on purified samples unless the sample contains only very few amounts of target RNA and the detection can become stochastic.

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Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular virology and diagnostics.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.
