# Asymptomatic screening of SARS-CoV-2 (COVID-19) virus RNA using reverse transcriptase loop-mediated isothermal amplification (RT-LAMP)

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**Method Article**

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# Abstract

This study demonstrates the diagnostic performance of SARS-CoV-2 RT-LAMP assays, comparing the performance of genomic versus sub-genomic sequence target with subsequent application in an asymptomatic screening population. An RT-LAMP workflow was developed using synthetic positive control RNA and the diagnostic sensitivity and specificity was then determined using clinical patient samples processed through the diagnostic RT-PCR service within the University Hospitals of Leicester NHS Trust. 92 RT-PCR clinically positive and 88 RT-PCR clinically negative swab samples along with 78 clinically positive and 63 clinically negative saliva samples were equally detected at 100% DSe and 100% DSp for all samples reporting a Ct < 20. DSe for all samples reporting a Ct < 30 reduced slightly to around 95% (100% DSp) for both the single genomic (large open reading frame; orf1a) and dual sub-genomic (nucleocapsid plus envelope) targeting RT-LAMP assays. Lastly, the diagnostic performance of a saliva direct workflow was only about 50% that of the saliva RNA extraction workflow.

Subsequently, a swab based RNA -RT-LAMP assay was implemented to ISO 15189:2012 standards supporting an advisory COVID-19 screening program for staff and students at the University of Leicester between October and December 2020. Within a 24-hour period, total nucleic acid extraction was followed by genomic target RT-LAMP plus an internal total RNA control to mitigate the possibility of false negative reporting. SARS-CoV-2 RT-LAMP positive samples were confirmed by an RT-PCR test in an NHS diagnostic laboratory and results were included within national statistics. Nine confirmed positive samples were detected in 1680 symptom free individuals (equivalent to 540 cases per 100,000) thus demonstrating the utility of RT-LAMP molecular diagnostic tool for the detection of SARS-CoV-2 in an asymptomatic population.


# Introduction

On the 31st December 2019, a cluster of cases of pneumonia were reported in Wuhan, the capital of central China's Hubei province. Novel coronavirus disease 2019 (COVID-19) was eventually identified, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Early availability of the complete genome of SARS-CoV-2 (Wuhan-Hu-1, GenBank accession number MN908947; Zhang 2019) followed by several other genomes deposited in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data (GISAID) facilitated the development of specific primers and standardized laboratory protocols for COVID-19 diagnosis to support quarantine efforts and prevent further spread [1, 2]. Corman *et al.,*[3] detailed the first real-time polymerase chain reaction (RT-PCR) assay protocols targeting the RNA-dependent RNA polymerase (RdRp gene of the open reading frame; Orf1ab sequence), envelope (E), nucleocapsid (N) and spike (S) gene of SARS-CoV-2. Assays targeting the E gene and RdRp gene demonstrated highest sensitivity (5.2 and 3.8 copies per reaction at 95% detection probability, respectively) and were subsequently followed by a variety of assays by different manufacturers targeting one or more RNA gene targets [4]. According to Government guidance published by the Medicines and Healthcare Products Regulations Agency (Target Product Profile for Laboratory-Based SARS-CoV-2 Viral Detection tests), a dual (or more) target SARS-CoV-2 RNA format is desirable, but

use of a single target is acceptable. Dual target assays protect against false-negative results caused by genome sequence mutations in the assay target sites and can offer improved certainty in results when the results of both targets are in agreement, but interpretation of results can be complicated where the results are discrepant. The interpretation and assessment of clinical significance of positive SARS-CoV-2 RT-PCR results can be equally challenging (PHE guide 'Understanding cycle threshold' (Ct) in SARS-CoV-2 RT-PCR'). A positive result with low viral load (high Ct) can be seen in both the early stages of infection (before the person becomes capable of transmission) or later in infection when transmission risk is low [5]. If therefore, the purpose of a public health utility test is to identify individuals who are currently infectious then data from highly sensitive RT-PCR needs careful interpretation.

Mass demand and delays associated with centralised RT-PCR testing and reagent availability were soon recognised as major obstacles in effectively responding to the SARS-CoV-2 pandemic with mitigation strategies urgently required [6]. Use of an alternative isothermal reverse transcriptase loop mediated amplification (RT-LAMP) strategy [7] with its completely different design, equipment and reagent requirements could largely bypass some of the challenges detailed above. Reactions comprise of 4-6 primers targeting 6-8 template region, typically spanning in excess of 200 bp. Therefore, amplification and confirmation of a positive result is only achieved with good quality RNA more suggestive of the presence of intact viral particles associated with active infection. Detection of slowly degrading RNA fragments, of historical no longer contagious infection, is less likely with RT-LAMP than with conventional RT-PCR designed to amplify significantly shorter sequence regions.

Utilizing a strand-displacing polymerase with reverse transcriptase activity, formation of stem loops permits fast amplification, which can be detected by a variety of endpoint readouts including fluorescence, turbidity and colorimetric change optimal for point of care LAMP-based diagnostics [8]. Several groups demonstrated the suitability of an RT-LAMP detection strategy early on in the pandemic. Rabe and Cepko [9] optimised primers directed toward a non-conserved region of the SARS-CoV-2 Orf1a gene, with subsequent inclusion of poly-T linkers within forward and backward inner primers (FIP and BIP) for enhanced reaction velocity (assay termed Orf1a-Harvard Medical School enhanced or 'Orf1a-HMSe'). Similarly, New England Biolabs (NEB) responded with the development of a dual sub-genomic assay targeting regions of the N and E gene, plus a separate internal control assay targeting the human beta actin gene (ACTB) for confirmation of total RNA indicative of appropriate sample collection [10]. Fowler *et al.,* [11] proceeded to optimise and validate OptiGene's COVID-19 RT-LAMP kits (targeting the Orf1ab) successfully establishing the first CE-IVD registered RT-LAMP workflow, now employed nationally across several NHS Trusts. Subsequently the method was further optimised for direct detection of SARS-CoV-2 within saliva [12, 13] made possible due to RT-LAMPs inherent tolerance to the various components of clinical samples in contrast to RT-PCR [14] and also the demonstrated stability of RNA in saliva without preservatives at temperatures up to 30°C for prolonged periods [15]. Furthermore, saliva offers potential advantages over conventional ON swabs as a less intrusive process [16, 17, 18].

The work presented herein compares the choice of SARS-CoV-2 RT-LAMP targets (genomic versus sub-genomic), end-point readout (colorimetric versus fluorescent detection), sample collection matrix (ON swabs versus saliva on extracted RNA) and finally assesses the performance of a saliva direct workflow. Assay limit of detection was confirmed using synthetic SARS-CoV-2 positive control RNA from Twist Bioscience of known concentration, and evaluation of diagnostic sensitivity (DSe) and specificity (DSp) was confirmed using residual RNA from UHL NHS inpatient oropharyngeal / nasopharyngeal (ON) swab samples (with corresponding RT-PCR Ct value) and saliva collected under ethics from UHL NHS inpatients. A swab based fluorescent end-point RT-LAMP reaction targeting the genomic Orf1a with parallel total RNA internal control reaction to mitigate reporting of false negative results subsequently provided the basis of an asymptomatic screening programme available to all staff and students at the University of Leicester.

# Materials And Methods

**Ethics Statement**: UHL NHS patient ON swab samples used in this study were collected in the context of routine clinical patient care. RT-LAMP analyses performed on residual de-identified patient material served as post-RT-PCR assay validation. Fast track ethical approval was obtained saliva tests allowing us to assess the performance of saliva direct RT-LAMP (no RNA extraction) against RT-LAMP on extracted RNA from matched saliva sample. (REC reference: 20/EE/0212).

### University of Leicester Asymptomatic Screening Programme

Participants wishing to access the COVID-19 screening programme required registration at the primary care Leicester Victoria Park Health Centre in order to generate a pathology request form. Participants self-swabbed (throat and lower nasal cavity) at a supervised screening venue using Miraclean swabs placed into PrimeStore Molecular Transport Medium for viral inactivation and RNA stabilisation at room temperature (Longhorn Vaccines and Diagnostics). Within a 24-hour period, total nucleic acid extraction was followed by RT-LAMP (65°C for 20 minutes) targeting the Orf1a (Orf1a-HMSe primer design) plus an internal ACTB control (NEB primer design). Data was uploaded to the pathology iLab system and NHS laboratory RT-PCR testing confirmed any positive RT-LAMP results, feeding into the national track and trace system. Results were reported back to participants (< 48 hours post sample collection) via SMS (for negative result) or phone call (for positive results) from the Victoria Park Health Centre. Figure 1 details the programme workflow.

**Control RNA**: Synthetic SARS-CoV-2 RNA at a concentration of $1\times10^6$ RNA copies per microliter was purchased from Twist Bioscience and diluted appropriately in nuclease free water (Twist Synthetic SARS-CoV-2 RNA Control 1 [MT007544.1] - SKU: 102091 and Control 2 [MN908947.3] - SKU: 102024). Negative control RNA from related Betacoronavirus 1 (Strain OC43) and non-related Influenza A (H1N1) was purchased from ATCC. Total human RNA purchased from Invitrogen (4307281).

### Swab sample collection and RNA extraction

Standard ON swabs from hospital inpatients were collected using PHE-approved flocked swabs placed into viral transport media (Virocult / VTM-M4RT). RNA extraction (on 200 µl of inactivated sample mixed with 265 µl Binding Solution) was carried out using the MagMAX Viral/Pathogen Nucleic Acid Isolation kit (MVPII, Thermofisher) on the KingFisher Flex Purification System. Samples were stored at 4°C short term prior to RT-LAMP and diagnostic RT-PCR confirmation at the University Hospital Leicester NHS Trust (UHL).

### Saliva Collection and RNA extraction

Research nurse assisted UHL inpatient saliva sample collection was performed at least 30 minutes after a meal, taking of oral medication or brushing of teeth. Pre-collection, patients rinsed their mouth with water and then waited 10 minutes before up to 5 ml of saliva was collected in universal collection vessels and stored at 4°C for up to three days prior to RNA extraction (as detailed above). Purified RNA was stored at -20°C prior to RT-LAMP (performed using the Rotor-Gene Q platform) or RT-PCR processed through the diagnostic service in the University Hospitals of Leicester NHS Trust.

### NHS real-time RT-PCR

Purified nucleic acid from ON swab samples or saliva was reverse transcribed into cDNA and amplified using a CE marked, locally validated commercially available kit targeting the E and S-gene sequence regions (RealStar® SARS-CoV-2 RT-PCR kit, Altona Diagnostics GmbH, Hamburg, Germany). Residual RNA from samples processed through the diagnostic RT-PCR service in the UHL NHS Trust with corresponding Ct value were frozen at -80°C until required for validation of RT-LAMP.

### RT-LAMP primers

Primers targeting the Orf1a (Orf1a-HMSe) were designed by Rabe and Cepko [9]. Primers targeting the nucleocapsid (N), envelope gene (E) and internal human beta-actin internal control (ACTB) were designed by NEB [10]. Primer sequences are listed in supplementary Table 3. HPLC grade purification primers were synthesised by Merck and reconstituted in nuclease free water. Individual RT-LAMP primer sets were prepared as 20 times final concentration stock, with final assay concentrations of 0.2 µM F3/B3, 1.6 µM FIP/BIP and 0.4 µM LoopF/Loop B.

### Fluorescent RT-LAMP

Reactions contained 1 X WarmStart® LAMP Master Mix (E1700) supplemented with 1 X fluorescent dye (NEB dye provided with E1700 master mix), 0.02 U/µL Antarctic Thermolabile UDG (NEB), 700 µM Thermolabile dUTP (NEB) and 1 X standard concentration LAMP primers. Unless otherwise stated, reactions were prepared to a final reaction volume of 25 µl using nuclease free water and incubated at 65°C using a either a StepOnePlus (Applied Biosystems) or Rotor-Gene Q (Qiagen) thermoclycler for up to 30 cycles (unless otherwise stated), with signal acquisition every 60 s.

### Colorimetric RT-LAMP

Reactions contained 1 X WarmStart® Colorimetric LAMP Master Mix (M1800; NEB) supplemented with 1 x EvaGreen (Biotium), 0.02 U/µL Antarctic Thermolabile UDG (NEB), 700 µM Thermolabile dUTP (NEB), 1 X standard concentration LAMP primers and 40 mM guanidine chloride solution (Sigma G3272, pH adjusted to pH ~ 8). Reactions were prepared to final volume of 25 µl using nuclease free water, incubated at 65°C for 30 minutes on a StepOnePlus thermocycler (Applied Biosystems). The colour of finished reactions was recorded using an office flatbed scanner.

### Saliva direct RT-LAMP

On the day of sample collection, 50 µl of saliva was mixed with 50 µl of MicroLYSIS buffer (Clent Scientific), heated at 95°C for 10 minutes to achieve viral inactivation, placed on ice and processed via fluorescent RT-LAMP in under 20 minutes. All saliva assays were performed using the Rotor-Gene Q platform.

### Statistical analysis

Time to positive (TTP in minutes) served as a surrogate for RT-PCR Ct and a semi-quantitative measure of viral RNA concentration. Additional product specificity checks were provided by melt curve analysis within acceptance range (2 degrees either side of the mean Tm determined during assay validation using patient samples). Regression calculations determining reaction efficiency incorporated data from concentrations where three or more values reported a TTP in under 25 minutes. Reactions were considered as negative with a TTP above 25 min. Reaction efficiency is calculated by $E=-1+10^{(-1/\text{slope})}$. Validation data using synthetic material represent the average of two independent experiments, performed in quadruplicate, presented as mean TTP ± S.E.M. Validation data using residual patient RNA is a single reaction performed in parallel reactions targeting the Orf1a, N + E and ACTB total RNA control sequence. MedCalc® Scientific Software was used for diagnostic test evaluation to determine the diagnostic sensitivity (DSe) referring to the proportion of known positive samples that tested positive in the assay and diagnostic specificity (DSp) referring to the proportion of samples from known negative reference samples that test negative in the assay.

## Results

**Genomic versus sub-genomic RT-LAMP assay target validation.** The performance of fluorescent and colorimetric end-point RT-LAMP reactions targeting the genomic Orf1a and sub-genomic N and E gene regions were tested solo, in duplex (N+E) and in multiplex combination (Orf1a+N+E) against a single concentration of two synthetic positive control RNAs (Figure 2). Fluorescent end-point data presented in Figure 2A demonstrates equivalent amplification of the Twist positive controls (T1 and T2) with no statistically significant difference. Reactions targeting the Orf1a were fastest to exceed the threshold (17 ± 0.17 and 16.7 ± 0.17 minutes, T1 and T2 respectively), followed by reactions targeting the E gene (12.1 ± 0.09 and 12.4 ± 0.10 minutes) and finally the N gene (17 ± 0.17 and 16.7 ± 0.17 minutes). Dual N+E reactions exceeded the amplification threshold at a mid-point between N and E alone (14.3 ± 0.10 and

14.2 ± 0.08 minutes). Finally, addition of the Orf1a primer set to the dual N+E reaction further enhanced dual velocity (13.4 ± 0.22 and 13.0 ± 0.21 minutes). The total RNA control reaction (ACTB) failed to amplify the positive RNA controls consistent with these being synthetic material. All SARS-CoV-2 targeting reactions also failed to amplify total human RNA, related human coronavirus OC43 and non-related influenza A. Presence of viable RNA within these samples was confirmed by control ACTB amplification (8.9 ± 0.25, 13.0 ± 0.19 and 9.5 ± 0.09 respectively). Finally, no template controls (NTC) confirmed the absence of non-specific amplification in any reactions. Fluorescent data is summarised in Tale 1. Summary melt curve data was also collated for each primer set (Orf1a 82.1 ± 0.058, N+E 89.4 ± 0.060, ACTB 92.7 ± 0.050), and used as an amplification product specificity check in subsequent analyses of patient samples. End-point colorimetric detection (Figure 2B) consistent with fluorescent findings, demonstrated clear amplification in under 30 minutes with a colour change from pink to yellow for all primers (Orf1a, N and E) and primer combinations (N+E & Orf1a+N+E) against positive RNA controls T1 and T2. Amplification by the ACTB internal control reaction was absent against the synthetic positive controls. Finally, a lack of cross-reactivity of all SARS-CoV-2 targets was confirmed by a lack of amplification in negative control wells OC43, influenza A and human RNA. No amplification was observed in NTC reaction wells.

**Limit of detection of fluorescent and colorimetric end-point RT-LAMP reactions targeting the Orf1a, dual N+E gene and multiplex Orf1a+N+E genes of SARS-CoV-2:** Parallel fluorescent and colorimetric end-point reactions were performed against synthetic RNA (Twist control 2) serially diluted to 10,000, 1,000, 500, 100, 50 and 10 copies of viral sequence. Representative fluorescent end-point amplification curves and linear regression analysis of primer sets Orf1a, N+E duplex and multiplex Orf1a+N+E target are presented in Figure 3 with summary data presented in Table 2. RT-LAMP targeting Orf1a was the fastest to exceed amplification threshold at 8.9 ± 0.12 minutes (10,000 viral RNA copies) and capable of reproducibly detecting 500 copies synthetic viral RNA. Lower viral loads down to 10 copies were detectable within 20 minutes although not reproducibly. RT-LAMP dual targeting N+E and multiplex Orf1a+N+E were ~5 minutes slower to exceed amplification threshold for equivalent viral loads, analogously capable of reproducibly detecting 500 copies of synthetic viral RNA. All assays demonstrated good linearity and efficiency, 102.1%, 99.5% and 105.5% respectively. Colorimetric reactions (supplementary Table 1) augmented with intercalating dye (EvaGreen) and guanidine hydrochloride were equally able to reproducibly detect 500 viral RNA copies, exceeding the amplification threshold slightly faster that their fluorescent counterpart (2.7, 5.6 and 4.5 minutes faster for Orf1a, N+E and Orf1a+N+E respectively for 500 viral copies). All reactions that exceeded the amplification threshold (as indicated by additional intercalating fluorescent dye TTP data) also showed a clear visual colorimetric change from pink to yellow in under 30 minutes.

**Diagnostic validation of fluorescent end-point RT-LAMP assays targeting Orf1a and dual N+E gene regions.** Residual RNA extracted from patient ON swab samples originally processed through the diagnostic RT-PCR service in the University Hospitals of Leicester NHS Trust (UHL) were processed through parallel RT-LAMP reactions targeting the Orf1a, dual N+E gene and ACTB internal control. Two PCR platforms were assessed: the ABI StepOnePlus PCR platform (114 UHL positive samples & 88 UHL

negative samples) and the Qiagen Rotor-Gene Q (39 UHL positive samples & 41 UHL negative samples). RT-LAMP reactions performed on the StepOnePlus platform with patient samples reporting an original RT-PCR of Ct < 20 were equally detected by Orf1a and N+E RT-LAMP at 100% / 100% DSe / DSp). DSe / DSp decreased slightly to 93.6% / 100% and 92.6% / 100% for the Orf1a and N+E targets respectively for patient samples reporting an RT-PCR Ct < 30 and then to 79.8 % / 100 % and 78.1 % / 100 % DSe / DSp respectively upon processing all patient samples reporting an RT-PCR Ct < 40 (Table 3A). When using the Rotor-Gene Q platform, RT-LAMP reactions performed with samples reporting an original RT-PCR of Ct < 20 were detected equivalently by Orf1a and dual N+E RT-LAMP reporting 100% / 100 % DSe / DSp; decreasing to 93.9 % / 100% and 97.0 % / 100 % DSe / DSp for samples reporting an RT-PCR Ct < 30 and finally to 84.3 % / 100% and 82.4 % / 100 % DSe / DSp (Orf1a and N+E respectively) for samples reporting an RT-PCR Ct < 40 (Table 3B). It is important to note that the residual patient RNA samples used for validation had been stored for some weeks, thus there may have been a degree of sample degradation. An additional product specificity check provided by the melt curve (Tm) confirmed selective amplification of the product.

**Diagnostic validation of Colorimetric RT-LAMP assays targeting Orf1a and dual N+E gene regions:** Residual RNA extracted from patient ON swab samples were also processed through colorimetric end-point RT-LAMP reactions targeting the Orf1a, dual N+E gene plus ACTB internal control reaction. Samples reporting an original RT-PCR of Ct < 30 were detected equally by Orf1a and N+E RT-LAMP assays (21 of 21 RT-PCR positive samples and 40 of 40 negative samples) demonstrating 100% DSe and DSp. Assay performance decreased to 85.7% / 100 % DSe / DSp for patient samples reporting an RT-PCR Ct < 40 (18 of 21 RT-PCR positive samples). Additional performance of a triple target (Orf1a+N+E) assay demonstrated 100% DSe and DSp (41 of 41 RT-PCR positive samples and 40 of 40 negative samples) decreasing to 90.2% / 100% DSe / DSp for samples reporting an RT-PCR Ct < 40 (37 of 41 RT-PCR positive samples). (Supplementary Table 2).

**Diagnostic validation of saliva RT-LAMP assays targeting Orf1a and dual N+E gene regions:** RNA-RT-LAMP reactions on samples reporting an original RT-PCR of Ct < 20 were equivalently detected by Orf1a and dual N+E RT-LAMP, reporting DSe / DSp of 100% / 100 %. Assay performance (DSe / DSp) decreased to 93.0 % / 100% and 91.6 % / 96.8 % (Orf1a and N+E respectively) for samples reporting an RT-PCR Ct < 30 and finally to 85.9 % / 100% and 84.6 % / 96.8 % for samples reporting an RT-PCR Ct < 40 (Table 4A). When testing the matched saliva-direct samples, RT-LAMP reactions on samples reporting an original RT-PCR of Ct < 20 were detected by Orf1a and dual N+E RT-LAMP at 51.7% / 100% and 41.4% / 100 % (DSe / DSp). Performance decreased to 40.9 % / 100% and 35.2 % / 100 % DSe / DSp for samples reporting an RT-PCR Ct < 30 and finally to 37.2 % / 100% and 33.3 % / 100 % DSe / DSp (Orf1a and N+E respectively) for samples reporting an RT-PCR Ct < 40 (Table 4B). An additional product specificity check provided by the melt curve (Tm) confirmed selective amplification of the product.

**University of Leicester RT-LAMP asymptomatic screening Programme:** RNA extracted from throat and lower nasal cavity swabs from 1,673 symptom free individuals attending campus in semester 1 autumn 2020 were processed through RT-LAMP targeting the Orf1a and internal control ACTB.Screening was

performed to ISO 15189:2012 standards as guided by the Leicester Molecular Diagnostic Lab, using NHS IT infrastructure and supported by University Hospital Leicester via confirmatory NHS diagnostic laboratory testing on any RT-LAMP 'positive' samples (feeding into national statistics). All results (negative and confirmed positive) were reported back to participants (< 48 hours post sample collection) through the GP Health Centre (SMS for negative results and phone call for positive result). During this period, a single repeat swab was requested from an individual due to inefficient sampling as identified by failure of amplification within the ACTB control reaction indicating absence of total RNA. In total, 9 RT-PCR confirmed RT-LAMP positive results from a total of 1,673 tests demonstrated a prevalence of asymptomatic infection of 0.54% (540 cases per 100,000). The presence of an internal control mitigated the risk of false negatives demonstrating the value and usability of RT-LAMP molecular diagnostic tool for the detection of SARS-CoV-2 in an asymptomatic population.

# Discussion

This report demonstrates selective amplification of SARS-CoV-2 viral RNA by RT-LAMP assays targeting genomic (Orf1a) and dual sub-genomic (N + E) RNA sequence regions via fluorescent or colorimetric determination. Equivalent sensitivity was observed for genomic (Orf1a) and dual sub-genomic (N + E) targets with assays capable of reproducibly detecting 500 copies of Twist Bioscience synthetic positive control RNA. RT-LAMP targeting the Orf1a was significantly faster to exceed amplification threshold due to enhanced reaction design and inclusion of a poly T linker within the FIP and BIP primer pairs facilitating faster loop formation [9]. Inclusion of guanidine chloride within colorimetric reactions also slightly enhanced amplification velocity compared to the fluorescent end-point counterparts [10].

Diagnostic validation of SARS-CoV-2 RT-LAMP reactions using RNA extracted from hospital inpatient ON swabs or saliva demonstrated the equivalent DSe and DSp (100% / 100%) of both genomic and dual sub-genomic target assays, concordant with comparator RT-PCR for Ct < 20 (fluorescent end-point analysis performed using the StepOnePlus and RototGene Q PCR platform with additional colorimetric end-point analysis of swab RNA samples). A small but comparative drop in DSe was observed for both swab and saliva RNA extraction assays targeting the Orf1a and N + E regions for all samples reporting an RT-PCR comparator Ct < 30. DSe across different PCR platforms decreased within range of 92.6% and 97.0% along with a slight reduction in DSp (96.83%) for RNA-RT-LAMP when running the N + E assay on the StepOnePlus (but not when running the RotorGene 5). No clear significant difference in DSe was observed between the Orf1a or N + E target assays for RNA extracted from swab or saliva samples.

Upon inclusion of all samples reporting a comparator RT-PCR Ct < 40, both Orf1a and N + E RT-LAMP assays performed on RNA extracted from saliva or swabs remained comparable (DSe within range of 82.4% and 85.9%; 100% DSp) when comparing like-for-like RotorGene Q PCR platform. For assays performed on the StepOnePlus platform, DSe dipped just below 80% (100% DSp). Interestingly, colorimetric triple target (Orf1a + N + E) RT-LAMP achieved higher sensitivity in patient sample validation, reporting DSe / DSp of 90.2% / 100% for all patient samples reporting a Ct < 40 (triple target fluorescent end-point not assessed due to lack of residual template RNA). These RT-LAMP findings for swab versus

saliva correlate highly (albeit lower overall sensitivity) with findings of Yee *et al.*, [19], reporting identical comparative positive percentage agreement (PPA) of swab and matched saliva in symptomatic adults (PPA of 93.8%) when at least one of three genes targeted (N, S or ORF1ab gene) fell under Ct < 40 (RT-PCR TaqPath COVID-19 Combo Kit).

Ultimately, the superior sensitivity harnessed by RT-PCR presents a well-documented drawback ubiquitous for inferring infectiousness from RT-PCR detection, with slowly degrading SARS-CoV, MERS, Influenza, Ebola and Zika viral RNA all detected long after the disappearance of the infectious virus [20]. An RT-PCR positive therefore reflects an assay's ability to detect viral RNA and not necessarily the presence of viable virus. The only robust way to detect viable virus is by cell culture, however this method is labour-intensive, slow and not amenable to high-throughput processing so is not suited to large-scale diagnostic or screening programmes. In comparison to RT-PCR, RT-LAMP assays are designed over a larger RNA template (> 200 bp) reducing the likelihood of detecting residual fragments of viral RNA. Pertinently the anonymised patient swab and saliva samples used to validate this study were not paired with clinical data detailing the duration from onset of symptoms, however, patients present to hospital a median of 7 to 10 days from onset of symptoms [21] at which point infectious virus may no longer be found despite ongoing detection of viral load by RT-PCR [20, 22]. Word by La Scola's group [23, 24] conducted RT-PCR testing and virus culture on positive samples with known Ct values showing that virus could not be isolated from samples collected after day eight of symptom onset, despite ongoing high viral loads. Only 70% of 3790 positive samples with Ct < 25 could be cultured, compared with less than 3% of the cases with Ct values above 35. In agreement, Bullard *et al.*, [25] took 90 SARS-CoV-2 RT-PCR–confirmed positive samples and demonstrated no viral growth in samples with a Ct > 24 or symptom onset to test time > 8 days suggesting the infectivity of patients with Ct > 24 and duration of symptoms > 8 days may be low. Current guidelines from the Centers for Disease Control and Prevention and World Health Organization also call for patients to isolate for 10 days after onset of symptoms, recognising that individuals are not likely to be infectious after that period. For these reasons (and assuming good sampling and sample handling) use of an assay of lower analytical sensitivity (or application of a cut-off in an assay of higher analytical sensitivity) may be preferable for an asymptomatic screening programme designed to detect potentially infectious individuals, to avoid 'false-positive' detection of residual RNA in individuals who have recovered from COVID-19.

Removal of the RNA extraction step and direct addition of consenting NHS inpatient saliva into RT-LAMP assays resulted in a considerable drop in DSe (maintaining 100% DSp), positively identifying only 51.7% and 51.4% (Orf1a and N + E respectively) of RT-PCR confirmed positives reporting a Ct < 20 and 41% and 35% (Orf1a and N + E respectively) of samples reporting a Ct < 30. This is perhaps unsurprising given the aforementioned viral culture data and the fact that viral loads in throat swab and sputum samples peak at around 5–6 days after symptom onset [26], most likely prior to these tests. Implementation of a saliva direct protocol earlier with the saliva Direct RT-LAMP (Optigene) workflow achieved DSe / DSp of 82.7% / 100% (Orf1a targeting), in a cohort inclusive of asymptomatic and symptomatic NHS healthcare staff plus symptomatic NHS patients [13].

On the basis of these findings and in-line with best documented practices at the time, implementation of a University of Leicester Asymptomatic COVID-19 RT-LAMP Screening Programme followed a swab based RNA-extraction with RT-LAMP assay targeting the Orf1a and a reaction cut off time equivalent to Ct < 30. In any asymptomatic population when the infection prevalence is low, test specificity must be sufficiently high to ensure an acceptable positive predictive value [27]. In contrast to other RT-LAMP COVID-19 diagnostic workflows, an internal total RNA control reaction was included for each sample, minimizing the chance of false negative reporting and consequently improving the negative predictive value of this assay. Carry-over contamination prevention reagents (dUTP and UGD from NEB) were also included as standard, minimizing a serious and known challenge associated with isothermal amplification strategies. Targeting of the genomic sequence, combined with product melt curve specify check and confirmation by NHS laboratory RT-PCR found a SARS-CoV-2 asymptomatic infection incidence of 0.54 % within this population. Given that at least 50% of new SARS-CoV-2 infections are estimated to originate from exposure to asymptomatic individuals [28] able to transmit the virus for an extended period [29] (perhaps longer than 14 days), this value is relatively low, suggesting that social distancing behaviors and the prolonged restrictions imposed on this population have been successful. Due to the potential of significant silent spread by asymptomatic persons [30–32] it is crucial that screening efforts such as the work described here are directed at those without symptoms in a targeted manner.

These data therefore support the use of RT-LAMP for the identification of SARS-CoV-2 within the early, pre-symptomatic / asymptomatic stage of infection when viral RNA is intact, to support quarantine of active/recent infection. RNA extracted from swab or saliva report equivalent RT-LAMP diagnostic sensitivity (Orf1a and N + E target regions) supporting saliva based testing as a suitable method for frequent, repeat community testing.

# Declarations


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Ethics approval: Fast track HRA & HCRW ethics approval for study title: Evaluation of reverse-transcription loop-mediated amplification (RT-LAMP) for the rapid detection of SARS-CoV-2 in saliva samples - a pilot study.

REC reference: 20/EE/0212

Protocol number: EDGE ID 135135

IRAS project ID: 287983.

Competing interests: The authors declare no competing interests.

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# Tables

| | TTP (minutes): | | | | | |
|---|---|---|---|---|---|---|
| **SARS-CoV-2 target:** | **Twist 1 (SKU 102019)** | **Twist 2 (SKU 102024)** | **OC43** | **Influenza A** | **Human RNA** | **NTC** |
| **Orf1a** | 9.1 ± 0.07 | 8.7 ± 0.16 | na | na | na | na |
| **N** | 17 ± 0.17 | 16.7 ± 0.17 | na | na | na | na |
| **E** | 12.1 ± 0.09 | 12.4 ± 0.10 | na | na | na | na |
| **N+E** | 14.3 ± 0.10 | 14.2 ± 0.08 | na | na | na | na |
| **Orf1a+N+E** | 13.4 ± 0.22 | 13.0 ± 0.21 | na | na | na | na |
| **ACTB** | na | na | 13.0 ± 0.19 | 9.5 ± 0.09 | 8.9 ± 0.25 | na |

**Table 1. RT-LAMP primer investigation:** Data summary of an average of 2 independent experiments each performed in quadruplicate, presented as mean TTP ± S.E.M. No amplification is noted as ‘na’.

| Synthetic positive control RNA | Fluorescent RT-LAMP Mean TTP (min) ± SEM (N) | | |
|---|---|---|---|
| Copies / reaction | Orf1a | N+E | Orf1a+N+E |
| 10,000 | 8.9 ± 0.12 (8/8) | 14.4 ± 0.16 (8/8) | 14.1 ± 0.12 (8/8) |
| 1,000 | 11.6 ± 0.49 (8/8) | 17.1 ± 0.77 (7/8) | 17.2 ± 0.50 (8/8) |
| 500 | 13.3 ± 0.65 (8/8) | 21.4 ± 2.51 (8/8) | 17.2 ± 0.29 (8/8) |
| 100 | 14.1 ± 0.0 (1/8) | 22.0 ± 1.44 (5/8) | 23.2 ± 1.66 (5/8) |
| 50 | 16.4 ± 3.91 (3/8) | na | 29.2 ± 0.0 (1/8) |
| 10 | 11.6 ± 0.0 (1/8) | na | 21.1 ± 0.85 (2/8) |
| NTC | na | na | na |

**Table 2. Limit of detection of fluorescent end-point RT-LAMP reactions targeting the Orf1a, dual N+E gene and multiplex Orf1a+N+E of SARS-CoV-2 using a serially diluted Twist Bioscience synthetic positive control RNA.** Data summary is an average of 2 independent experiments, performed in quadruplicate and presented as mean TTP ± S.E.M. Numeration in parentheses indicates the number of repeat reactions achieving the amplification threshold required to report a TTP. No amplification ('na').

A) **StepOnePlus; Applied Biosystems:**

| RT-LAMP target: | SARS-CoV-2 Orf1a | SARS-CoV-2 Dual N+E |
|---|---|---|
| Dse (95% CI) | 100.0% (91.6% to 100.0%) | 100.00% (91.6% to 100.0%) |
| DSp (95% CI) **Ct < 20** (N42) | 100.0% (95.9% to 100.0%) | 100.00% (95.9% to 100.0%) |
| Dse (95% CI) | 93.6% (86.6% to 97.6%) | 92.6% (85.3% to 97.0%) |
| DSp (95% CI) **Ct < 30** (N94) | 100.0% (95.9% to 100.0%) | 100.0% (95.9% to 100.0%) |
| Dse (95% CI) | 79.8% (71.3% to 86.8%) | 78.1% (69.4% to 85.3%) |
| DSp (95% CI) **Ct < 40** (N114) | 100.0% (95.9% to 100.0%) | 100.0% (95.9% to 100.0%) |

B) **Rotor-Gene Q; Qiagen**

| RT-LAMP target: | SARS-CoV-2 Orf1a | SARS-CoV-2 Dual N+E |
|---|---|---|
| Dse (95% CI) | 100.0% (76.8% to 100.0%) | 100.0% (76.8% to 100.0%) |
| DSp (95% CI) **Ct < 20** (N14) | 100.0% (91.4% to 100.0%) | 100.0% (91.4% to 100.0%) |
| Dse (95% CI) | 93.9% (79.8% to 99.3%) | 97.0% (84.2% to 99.9%) |
| DSp (95% CI) **Ct < 30** (N33) | 100.0% (91.4% to 100.0%) | 100.0% (91.4% to 100.0%) |
| Dse (95% CI) | 84.4% (76.0% to 90.6%) | 82.4% (73.9% to 89.1%) |
| DSp (95% CI) **Ct < 40** (N39) | 100.0% (95.9% to 100.0%) | 100.00% (95.9% to 100.0%) |

**Table 3. Diagnostic sensitivity (Dse) and specificity (DSp) of a single (Orf1a) and dual (N+E) SARS-CoV-2 fluorescent end-point RT-LAMP assay amplifying residual RNA extracted from clinical patient ON swab samples with comparator RT-PCR Ct values.** Reactions were performed at 65°C for 30 minutes on **A)** StepOnePlus PCR platform, against 114 UHL positive samples & 88 UHL negative samples. **B)** Rotor-Gene Q Qiagen PCR platform, against 39 UHL positive samples & 41 UHL negative samples. Sensitivity and specificity with 95% CI are shown for samples with a corresponding RT-PCR Ct < 20, Ct < 30 and Ct < 40. Calculations performed using the MedCalc Scientific Software.

**Saliva RNA-extraction RT-LAMP:**

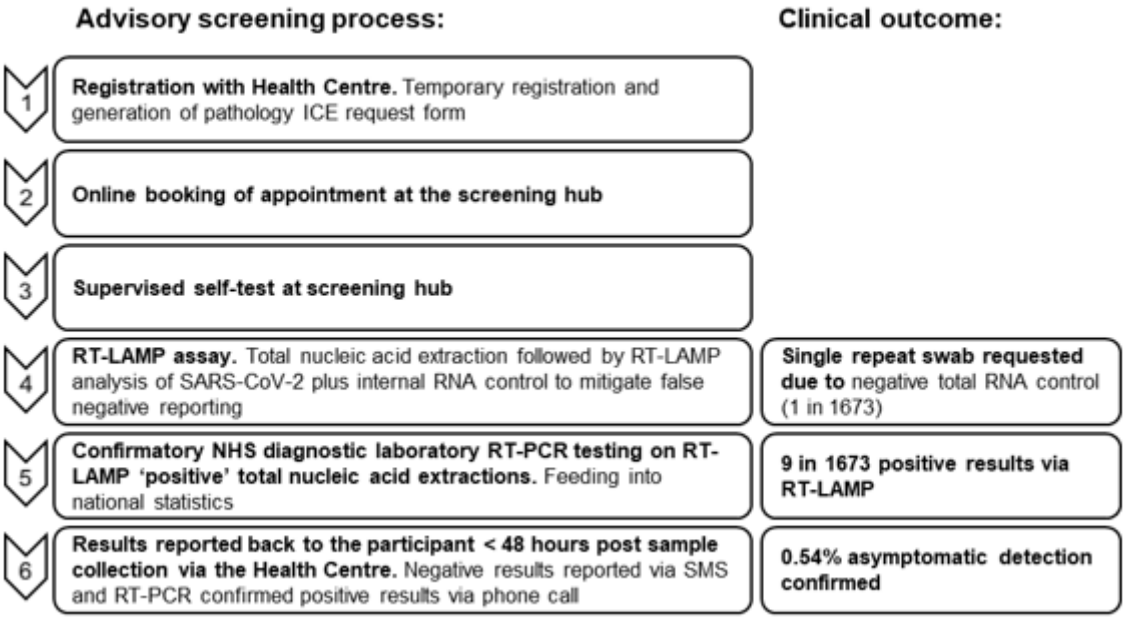
| RT-LAMP target: | SARS-CoV-2 Orf1a | SARS-CoV-2 Dual N+E |
|---|---|---|
| Dse (95% CI) | 100.0% (88.1% to 100.0%) | 100.00% (88.1% to 100.0%) |
| DSp (95% CI) **Ct < 20** (N29) | 100.0% (94.3% to 100.0%) | 100.00% (94.3% to 100.0%) |
| Dse (95% CI) | 93.0% (84.3% to 97.7%) | 91.6% (82.5% to 96.8%) |
| DSp (95% CI) **Ct < 30** (N71) | 100.0% (94.3% to 100.0%) | 96.8% (89.0% to 99.6%) |
| Dse (95% CI) | 85.9% (76.2% to 92.7%) | 84.6% (74.7% to 91.8%) |
| DSp (95% CI) **Ct < 40** (N78) | 100.0% (4.3% to 100.0%) | 96.8% (89.0% to 99.6%) |

**Saliva direct RT-LAMP:**

| RT-LAMP target: | SARS-CoV-2 Orf1a | SARS-CoV-2 Dual N+E |
|---|---|---|
| Dse (95% CI) | 51.7% 32.5% to 70.6% | 41.4% (23.5% to 61.1%) |
| DSp (95% CI) **Ct < 20** (N29) | 100.0% 94.3% to 100.0% | 100.0% (94.3% to 100.0%) |
| Dse (95% CI) | 40.9% (29.3% to 53.2%) | 35.2% (24.2% to 47.5%) |
| DSp (95% CI) **Ct < 30** (N71) | 100.0% 94.3% to 100.0% | 100.0% (94.3% to 100.0%) |
| Dse (95% CI) | 37.2% (26.5% to 48.9%) | 33.3% (23.1% to 44.9%) |
| DSp (95% CI) **Ct < 40** (N78) | 100.0% (94.3% to 100.0%) | 100.0% (94.3% to 100.0%) |

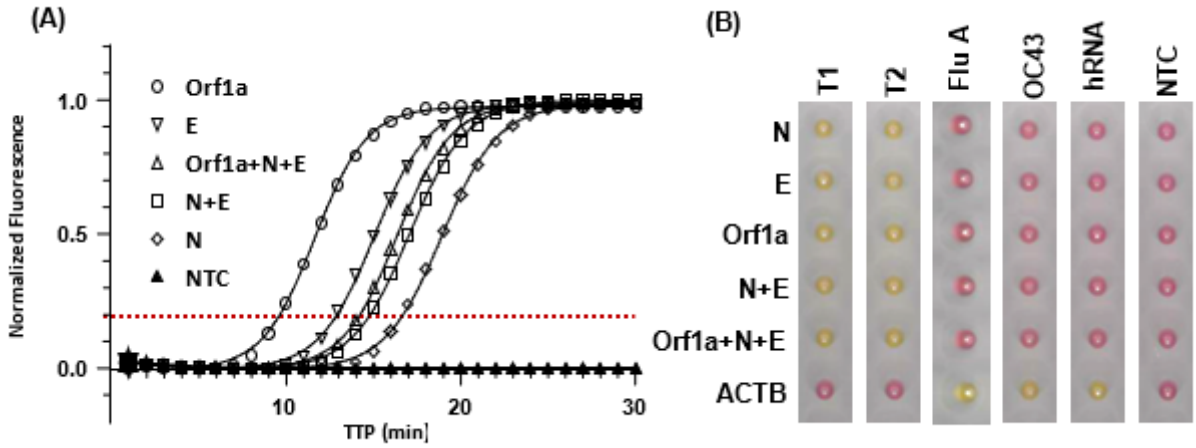
**Table 4. Diagnostic sensitivity (Dse) and specificity (DSp) of a single (Orf1a) and dual (N+E) SARS-CoV-2 fluorescent end-point RT-LAMP assay amplifying residual RNA extracted from clinical patient saliva samples with comparator RT-PCR Ct values.** Reactions were performed at 65°C for 30 minutes using **A)** RNA extracted from patient's saliva and **B)** viral inactivated saliva direct. All assays were performed using the Rotor-Gene Q Qiagen PCR platform, against 78 UHL positive samples & 63 UHL negative samples. Sensitivity and specificity with 95% CI are shown for samples with a corresponding RT-PCR Ct < 20, Ct < 30 and Ct < 40. Calculations performed using the MedCalc Scientific Software.

# Figures




## Figure 1

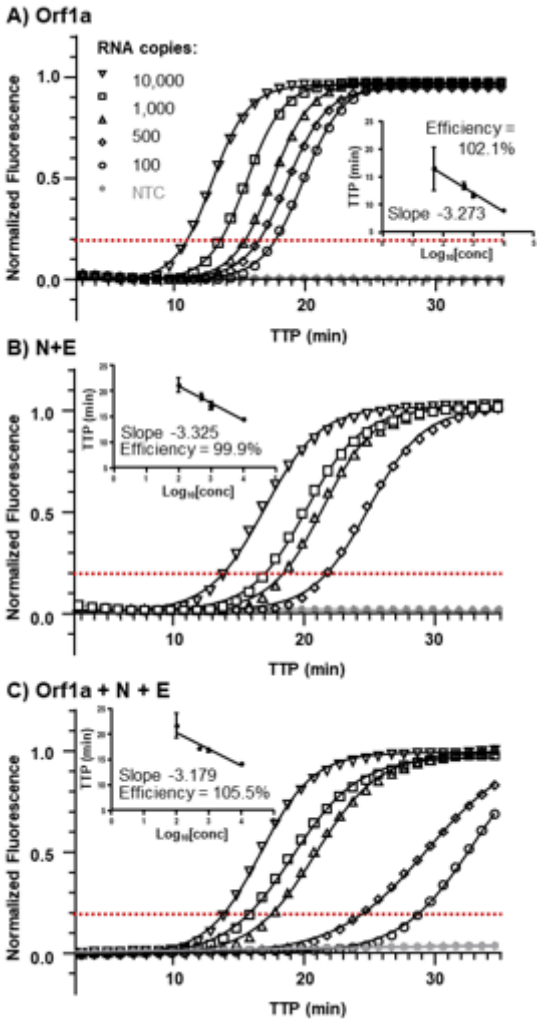
University of Leicester SARS-CoV-2 Advisory Screening Programme. Available to all students and staff without symptoms for a period of twelve weeks (October 2020 – December 2020) to allow rapid isolation and reduce outbreaks.




## Figure 2

RT-LAMP primer investigation: SARS-CoV-2 RNA primer sets targeting the nucleopcapsid (N), envelope gene (E) (NEB design) and Orf1a (Rabe and Cepko Harvard Medical School) were tested independently and in combination (dual N+E reaction and multiplex Orf1a+N+E reaction) against 1x104 copies of Twist synthetic SARS-CoV-2 control RNA (T1 and T2). Negative control RNA from Betacoronavirus 1 strain OC43 and Influenza A (H1N1) at a single concentration (1x105 copies per well) plus a water no template control (NTC). A total RNA control primer set (NEB) targeting human beta actin (ACTB) was also included

and tested against 5 ng total human RNA (hRNA). Both RT-LAMP fluorescent end-point and colorimetric 25 µl reactions were performed at 65°C for 30 minutes on a StepOnePlus thermocyler. (A) Representative fluorescent amplification curves where time to positive (TTP) is the time at which amplification exceeds the manually set, reaction consistent threshold (red dotted line). (B) Representative colorimetric reactions whereby yellow indicates positive amplification and pink no amplification.




## Figure 3

Limit of detection of fluorescent end-point RT-LAMP reactions targeting the Orf1a, dual N+E gene and multiplex Orf1a+N+E of SARS-CoV-2 using a serially diluted Twist Bioscience synthetic positive control RNA (control 2 GenBank ID MN908947.3, GISAID Wuhan-Hu-1). Synthetic RNA was serially diluted from stock to result in 10,000, 1,000, 500, 100, 50 and 10 copies of viral sequence per 25 µl reaction. Water no template control (NTC) were included in each reaction. Reactions were performed at 65°C for 40 minutes on the Qiagen Rotor-Gene Q Thermocycler platform. Representative amplification and linear regression analysis for each primer set are shown. (A) RT-LAMP targeting the Orf1a. (B) RT-LAMP targeting N+E duplex. (C) RT-LAMP triple Orf1a+N+E target. Time to positive (TTP) is the time at which amplification exceeds the manually set, reaction consistent threshold (red dotted line) when amplification enters the rapid linear, exponential phase. Data represents the average of two experiments each performed in

quadruplicate. Linear regression calculations incorporate data from concentrations whereby three of more values reported a TTP under 25 minutes. Reactions were considered as negative with a TTP above 25 min. Slope of linear regression and reaction efficiency (figure insets) whereby E=-1+10(-1/slope) are shown for each target reaction. No amplification ('na').

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AllsoppRASupplementaryInformation.docx