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Methods Article

RUNCOV: a one-pot triplex real-time RT-LAMP as a point-of-care diagnostic tool for detecting SARS-CoV-2

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Abstract

Given the risk of zoonotic disease emergence, including new SARS-CoV-2 variants of COVID-19, rapid diagnostic tools are urgently needed to improve the control of the spread of infectious diseases. A one-pot triplex real-time RT-LAMP (reverse-transcription-loop-mediated isothermal amplification) assay, based on two regions of the genome SARS-CoV-2—specifically the Orf1ab and N genes—along with one internal control, the human RNase P gene, was developed. The multiplexing relies on the distinct melting peaks produced during an annealing step. This tool, named RUNCOV, was compared to the gold-standard reverse-transcription real-time quantitative PCR (RT-qPCR) assay. A simple sample preparation step was designed alongside the assay, making it ready for use on site, as a point-of-care diagnostic tool. RUNCOV is rapid (typically less than 40 minutes), highly sensitive and specific. When tested on clinical samples with known SARS-CoV-2 status, its limit of detection (LOD) ranges between 5 and 20 copies per reaction and its diagnostic sensitivity (97.44%) and specificity (100%) values are high compared to the RT-qPCR gold standard. These results were supported with an extensive *in silico* analysis of over 14 million genomes, demonstrating this tool was capable of detecting all known SARS-CoV-2 variants, including the most recent ones KP.3.1.1 and BA2.86.1. This molecular assay is portable, as demonstrated when it was used successfully in La Réunion in different contexts outside the laboratory.

Keywords: SARS-CoV-2; triplex-RT-LAMP; detection; point-of-care testing.

Introduction

Since its emergence in 2019, the respiratory illness, Coronavirus disease 2019 (COVID-19), has caused 6.9 million deaths, with some 676.6 million cases globally, according to figures registered by 10/03/2023 (end of data collection by Johns Hopkins University COVID-19 Map—Johns Hopkins Coronavirus Resource Center (jhu.edu)). It has also severely affected public health systems. Although we are now in a post COVID-19 phase, the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), particularly the Omicron variant (GISAID—gisaid.org), is still circulating worldwide. Given the long-term consequences of the pandemic and the risk of emergence of new variants or new zoonotic diseases, rapid diagnostic tools are urgently needed to control the spread of infectious diseases. To this aim, numerous diagnostic tests have been developed and used intensely to control viral spread within the human population.

The gold-standard technique for detecting the genome of SARS-CoV-2 is a reverse-transcription real-time quantitative PCR (RT-qPCR) assay, known for its robustness and sensitivity. Various assays have already been developed [1], including multiplex assays targeting multiple genomic regions, along with an endogenous human control in a single reaction. Multiplex assays

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improve detection accuracy and are more likely to detect the virus in the case of mutations. However, RT-qPCR assays are limited because they require expensive laboratory equipment, high sample purity, and are relatively time-consuming. In contrast, point-of-care (POC) diagnostic assays, which can be conducted on-site without sophisticated equipment, are gaining popularity in the fields of human, animal, and plant health. Loop-mediated isothermal amplification (LAMP), first described by Notomi [2], is a sensitive, rapid, and low-cost isothermal method of DNA amplification. It can be used with relatively crude samples. On the one hand, numerous RT-LAMP assay protocols have already been developed for COVID-19 diagnosis. Most have demonstrated high performance in terms of limit of detection (LOD), specificity, and sensitivity, positioning them between RT-qPCR and rapid antigen tests [3, 4]. They utilized various read-outs, such as endpoint colorimetric detection [5–9], fluorescence [10–12], along with the CRISPR/Cas system for lateral flow detection and high-throughput sequencing [13, 14]. Nevertheless, some LAMP protocols and commercial kits rely on the amplification of a single target gene within the SARS-CoV-2 genome, which can be problematic due to the virus's ability to mutate and evade detection. Others are designed to amplify multiple targets, but these are performed in separate reactions, effectively increasing the cost of the assay [3]. Performant multiplex-LAMP protocols assays are available, based on the use of differential fluorescence probes, which in some cases are integrated into microfluidic chips [3, 5, 15, 16]. However, these multicolour fluorescent-based protocols remain expensive, require an RNA purification step, and are often challenging to implement outside the laboratory. On the other hand, multiplexing LAMP assays based on melting curve temperatures are very simple and cost-effective. They require a standard LAMP mix including a dsDNA-binding dye and a portable LAMP instrument equipped with at least one fluorescence channel [10]. To our knowledge, only Oscorbin *et al.* [17] have optimized a SARS-CoV-2 multiplex-LAMP assay based on a single viral target, including a synthetized internal control added to each sample before analysis.

Internal controls provide greater confidence in true-negative results by confirming that sampling was properly conducted, nucleic acid was successfully transferred to the LAMP reaction, and amplification occurred. These controls can be assessed in a separate reaction with SARS-CoV-2 primers, with the limitations mentioned above, or in the same reaction, such as RNaseP or actin amplification duplexed with SARS-CoV-2 primers using two-colour fluorescence detection [4].

In this study, we developed a real-time triplex RT-LAMP assay, RUNCOV, which is rapid, low-cost, highly sensitive, capable of detecting SARS-CoV-2 using a classical dye-LAMP mix, and relies on melting curve temperature discrimination without requiring an RNA extraction step. RUNCOV amplifies in one reaction, two genes -Orf1ab and N—of SARS-CoV-2 genome, and the human RNase P gene as an internal control. A rapid RNA preparation step was used to inactivate the virus before the RT-LAMP assay and successfully applied in clinical diagnosis using swabs from human patients. RUNCOV has been used for POC testing in La Réunion (France) at different sites, including the entrance to a hospital, the international airport, and private testing centres.

Materials and methods

RNA extraction and real-time quantitative PCR assay

RNA was extracted from 150-µL swab samples, eluted in a final volume of 50 µl using the NucleoSpin® RNA Virus Kit (Macherey Nagel, France). Five microlitres were tested by quantitative real-time reverse-transcription PCR (RT-qPCR) on a Quantstudio 5 instrument (Thermo Fisher, France), using the superscript III Platinum mix (Invitrogen, France) with the primer/Taqman® probe systems IP2 and IP4, according to the protocol developed by the French reference laboratory, the Pasteur Institute, Paris (https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2) (LOD = 80 copies/reaction for each viral target according to an internal evaluation (data not shown)).

LAMP primer screening

Eight published primer sets, together with the three new primer sets described in this study, were evaluated (Table 1). The three original LAMP primer sets were designed from the SARS-CoV-2 reference genome (Genbank NC_045512.2), using the Software Primer Explorer v5 (Eiken, Japan). All LAMP primers purified using the Oligonucleotide Purification Cartridge (MOPC™) method with ≥85% purity guarantee were supplied by Macrogen. The LAMP primer sets were evaluated on 10-fold dilutions of RNA extracted from a human patient's clinically positive rhinopharyngeal sample. The RNA extracts were also tested using the reference real-time qPCR, as described above. The LAMP primer sets with the highest sensitivity were screened based on the annealing temperatures (Ta, similar to a melting temperature (Tm)) of the amplified products in order to combine them in a single assay targeting two different regions of the viral RNA. Combinations of Ta compatible SARS-CoV-2 LAMP primer sets were evaluated in standard simplex LAMP conditions (see § 2.3.). Duplex RT-LAMP assays showing low or no amplification were discarded. The screened duplex SARS-CoV-2 assays were tested in triplex assays with an endogenous control, targeting the human *RNase P* [18]. This RNase P LAMP primer set was first tested on three negative saline swabs in the conditions described in § 2.7., to verify the efficiency of the LAMP reaction at different concentrations: 1X (=standard LAMP conditions, see § 2.3.) and 0.25X, 0.3X, 0.5X. The RNase P LAMP responses in the simplex assay were compared to those obtained with the final triplex RUNCOV protocol (§ 2.3.). The RUNCOV protocol was also run with or without the RNase P LAMP primers on three positive samples to evaluate the impact of RNase P LAMP primers on viral detection.

RT-LAMP reactions

Simplex RT-LAMP assays were performed with a portable real-time fluorescence reading device (Genie II, OptiGene, Horsham, UK), in a 25 µL total reaction volume, containing 15 µL ISO-DR004-RT Isothermal Mastermix (OptiGene, Horsham, UK), 0.05 µL of *amv* reverse transcriptase (Promega, France), 2.5 µL of pre-primer mix, giving final concentrations of 0.2 µM of each F3 and B3 primer, 0.8 µM of each Forward Inner Primer (FIP) and Backward Inner Primer (BIP), 0.4 µM of each loop primer, 2.45 µL nuclease-free water (Ambion™, Thermo Fisher Scientific, Waltham, MA, USA), and 5 µL of template RNA. These final standard primer concentrations were referred to as "1X". LAMP reactions were run at 65°C for 25 min, followed by an annealing step with temperatures from 98°C to 78°C at a speed of 0.05°C/second. Duplex RT-LAMP assays were performed in the same conditions except that the mix received 2.475 µL of each of the two LAMP primer sets (≃ 1X each). The final concentration of the RNase P primer set was 0.25X for the triplex RT-LAMP assays, associated with 1.5X $Orf8_{\text{-This-study}}$/0.9X $Nos2_{\text{-This-study}}$ or 1.4X $Nsp3_{\text{-Park}}$/0.9X $Nos2_{\text{-This-study}}$ or 2X $Orf1a_{\text{-Lamb}}$/0.8X $Nos2_{\text{-This-study}}$.

Table 1. LAMP primer sets used in this study.

Primer sets		Sequences	Target	Source
Orf1a_$_{\text{LAMB}}$	F3	TCCAGATGAGGATGAAGAAGA	*Orf1a*	Lamb *et al.*,[7]
	B3	AGTCTGAACAACTGGTGTAAG		
	FIP	AGAGCAGCAGAAGTGGCACAGGTGATTGTGAAGAAGAAGAG		
	BIP	TCAACCTGAAGAAGAGCAAGAACTGATTGTCCTCACTGCC		
	LoopF	CTCATATTGAGTTGATGGCTCA		
	LoopB	ACAAACTGTTGGTCAACAAGAC		
Orf1ab_$_{\text{SONG}}$	F3	TGCTTCAGTCAGCTGATG	*Orf1a* and *Orf1ab*	Song *et al.* [9]
	B3	TTAAATTGTCATCTTCGTCCTT		
	FIP	TCAGTACTAGTGCCTGTGCCCACAATCGTTTTTAAACGGGT		
	BIP	TCGTATACAGGGCTTTTGACATCTATCTTGGAAGCGACAACAA		
	LoopF	CTGCACTTACACCGCAA		
	LoopB	GTAGCTGGTTTTGCTAAATTCC		
Nsp3_1-61_$_{\text{PARK}}$	F3	GGAATTTGGTGCCACTTC	*Nsp3*	Park *et al.* [8]
	B3	CTATTCACTTCAATAGTCTGAACA		
	FIP	CTTGTTGACCAACAGTTTGTTGACTTCAACCTGAAGAAGAGCAA		
	BIP	CGGCAGTGAGGACAATCAGACACTGGTGTAAGTTCCATCTC		
	LoopF	ATCATCATCTAACCAATCTTCTTC		
	LoopB	TCAAACAATTGTTGAGGTTCAACC		
Nsp3_$_{\text{PARK}}$[a]	**F3**	**TGCAACTAATAAAGCCACG**	*Nsp3*	Park *et al.* [8]
	B3	**CGTCTTTCTGTATGGTAGGATT**		
	FIP	**TCTGACTTCAGTACATCAAACGAATAAATACCTGGTGTATACGTTGTC**		
	BIP	**GACGCGCAGGGAATGGATAATTCCACTACTTCTTCAGAGACT**		
	LoopF	**TGTTTCAACTGGTTTTGTGCTCCA**		
	LoopB	**TCTTGCCTGCGAAGATCTAAAAC**		
Spike_$_{\text{PARK}}$	F3	CTGACAAAGTTTTCAGATCCTCAG	*Spike*	[8]
	B3	AGTACCAAAAATCCAGCCTCTT		
	FIP	TCCCAGAGACATGTATAGCATGGAATCAACTCAGGACTTGTTCTTACC		
	BIP	TGGTACTAAGAGGTTTGATAACCCTGTTAGACTTCTCAGTGGAAGCA		
	LoopF	CCAAGTAACATTGGAAAAGAAA		
	LoopB	GTCCTACCATTTAATGATGGTGTTT		
N_$_{\text{PARK}}$	F3	GCCAAAAGGCTTCTACGCA	*N*	Park *et al.* [8]
	B3	TTGCTCTCAAGCTGGTTCAA		
	FIP	TCCCCTACTGCTGCCTGGAGGCAGTCAAGCCTCTTCTCG		
	BIP	TCTCCTGCTAGAATGGCTGGCATCTGTCAAGCAGCAGCAAAG		
	LoopF	TGTTGCGACTACGTGATGAGGA		
	LoopB	ATGGCGGTGATGCTGCTCT		
Orf1ab_$_{\text{ZHANG}}$	F3	CTGCACCTCATGGTCATGTT	*Orf1a*	Zhang and Tanner [36]
	B3	AGCTCGTCGCCTAAGTCAA		
	FIP	GAGGGACAAGGACACCAAGTGTATGGTTGAGCTGGTAGCAGA		
	BIP	CCAGTGGCTTACCGCAAGGTTTTAGATCGGCGCCGTAAC		
	LoopF	CCGTACTGAATGCCTTCGAGT		
	LoopB	TTCGTAAGAACGGTAATAAAGGAGC		
N_$_{\text{ZHANG}}$	F3	TGGCTACTACCGAAGAGCT	*N*	Zhang and Tanner [36]
	B3	TGCAGCATTGTTAGCAGGAT		
	FIP	TCTGGCCCAGTTCCTAGGTAGTCCAGACGAATTCGTGGTGG		
	BIP	AGACGGCATCATATGGGTTGCACGGGTGCCAATGTGATCT		
	LoopF	GGACTGAGATCTTTCATTTTACCGT		
	LoopB	ACTGAGGGAGCCTTGAATACA		
Spir_$_{\text{THIS_STUDY}}$	F3	CTGACAAAGTTTTCAG	*Spike*	This study
	B3	GTACCAAAAATCCAGCCTC		
	FIP	CCAGAGACATGTATAGCATGGAACCAACTCAGGACTTGTTCTTACC		
	BIP	GACCAATGGTACTAAGAGGTTTGATTTAGACTTCTCAGTGGAAGC		
	LoopF	GTACCAAAAATCCAGCCTC		
	LoopB	AACCCTGTCCTACCATTTAATGATG		
Nos2_$_{\text{THIS_STUDY}}$	**F3**	**TGGACCCCAAAATCAGCG**	*N*	This study
	B3	**GCCTTGTCCTCGAGGGAAT**		
	FIP	**CCATTCTGGTTACTGCCAAATGCACCCCGCATTACG**		
	BIP	**CGCGATCAAAACAACGTCGGCCCTTGCCATGTTGAGTGAGA**		
	LoopF	**TTGAATCTGAGGGTCCACCAA**		
	LoopB	**CCCAATAATACTGCGTCTTGGT**		
Orf8_$_{\text{THIS_STUDY}}$	F3	CCAAGAATGTAGTTTACAGTCAT	*Orf8*	This study
	B3	CCTGGCAATTAATTGTAAAAGGTA		
	FIP	TGCTGATTTTCTAGCTCCTACTCTACATCAACCATATGTAGTTGATGAC		
	BIP	CCTTTAATTGAATTGTGCGTGGATCAGGAAACTGTATAATTACCGATA		
	LoopF	GAATAGAAGTGAATAGGACACGG		
	LoopB	GCTGGTTCTAAATCACCCATT		
RNase P	**F3**	**TTGATGAGCTGGAGCCA**	*Rnase P*	Curtis *et al.* [18]
	B3	**CACCCTCAATGCAGAGTC**		
	FIP	**GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC**		
	BIP	**CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC**		
	LoopF	**ATGTGGATGGCTGAGTTGTT**		
	LoopB	**CATGCTGAGTACTGGACCTC**		

[a] The LAMP primer sets in bold are those selected for the final triplex RT-LAMP.

The optimal concentrations for the triplex RT-LAMP (RUNCOV) was 1.5X $Nsp3_{-Park}$/1.4X $Nos2_{-This-study}$/0.25X RNase P. The 25.5 µL total volume contained 15 µL of ISO-DR004-RT Isothermal Mastermix (OptiGene), 0.5 µL (7.5 units) of Warm start reverse transcriptase (NEB, UK), 1.24 µL of 28X primer pre-mix $Nos2_{-This-study}$, 2.48 µL of 15X primer pre-mix $Nsp3_{-Park}$ and 1.24 µL of 5X primer pre-mix RNase P—resulting in final concentrations of 0.29 µM, 0.27 µM, 0.05 µM of each F3 and B3 primers, 1.17 µM, 1.09 µM, 0.19 µM of each FIP and BIP primers, 0.58 µM, 0.55 µM, 0.05 µM of each LF and LB primers, for $Nos2_{-This-study}$, $Nsp3_{-Park}$ and RNase P, respectively—and 5 µL of RNA template. LAMP reactions were run at 65°C in the same conditions as described for the simplex-LAMP assays. All RT-LAMP reactions were performed using a portable device (Genie II, OptiGene). Vacuum-dried and encapsulated wild-type SARS-CoV-2 RNA controls (RNAshells®, Twist, UK) [19] or inactivated wild-type SARS-CoV-2 RNA (Appolon Bioteck, France) were added to each run, as well as non-target controls (NTC, with pure water as a template). A sample was considered positive if (i) a TTR ("Time-To-Results") value ≤ 25 min was obtained and (ii) at least one of the specific Ta for the viral targets was observed, ranging between 84.5°C and 86°C for Orf1ab and 88.5°C and 90°C for N, with a fluorescence derivative value greater than 1800. For a negative test to be considered valid, the internal control should be amplified with a temperature annealing peak between 86.5°C and 88°C and a threshold of 1000 for the fluorescence derivative value. If no internal control or SARS-CoV-2 amplification was detected, the test was considered inconclusive.

By convention, a TTR value of 26 (one unit above the limit of detection) was assigned to samples showing no fluorescent signals for their visualization on the different graphs and for calculating mean values for the replicates.

Preparation of RNA samples for direct LAMP SARS-CoV-2 virus detection

Sample collection medium

Six positive qPCR clinical samples were collected in physiological serum, including 1X inactivation solution, i.e. 1% of a 100X inactivation solution ((250 mM Tris(2-carboxyethyl) phosphine (TCEP), 100 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH7 with NaOH)). These samples, which were stored at −80°C, were heated at 95°C for 5 min, and 10-fold diluted in physiological serum (0.09% sodium chloride in ultrapure water), phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH = 7.4) and AMIES (EswabR1, Labellians, France), and tested with the triplex-LAMP (three replicates per condition).

Optimization step

A first assay was performed on 30 samples (11 SARS-CoV- RT-qPCR-positive samples and 19 SARS-CoV-2 RT-qPCR-negative samples). Samples were collected in physiological serum, including 1% of a 100X inactivation solution, and heated at 95°C for 5 min. Eight of the 11 qPCR- positive samples were diluted in SARS-CoV-2 negative nasopharyngeal swab samples, to obtain lower viral loads. As the samples were collected in different contexts, the number of replicates per condition varied from one (positive clinical samples) to six (dilutions of positive samples in negative swabs). These samples were tested with the RUNCOV protocol (§ 2.3.). In parallel, as a second assay, the same 30 samples were heated further for 2 min at 95°C in the LAMP microtubes before being tested with RUNCOV.

Specificity

Analytical specificity was tested on RNA from several non-target viruses, including respiratory viruses that induce similar clinical symptoms and Alpha and Beta coronaviruses isolated from animals (bats, pigs and poultry) (Table 2). Specificity was also assessed through an extensive *in silico* analysis. The two target regions were extracted from an alignment of 14,786,600 genomes available on GISAID (01/10/2024) using the extractalign function from EMBOSS version 6.6.0 [20]. Genomes with NNNs or ambiguous bases in Nsp3 and Nos2 were filtered out, leaving 13,538,889 genomes. Non-redundant sequences based on the polymorphism of Nsp3 and Nos2 were retained using the rmdup command from seqkit version 2.0.0 [21], resulting in 35,364 unique alleles. Primers were positioned, and the % identity was calculated with the reference sequence. Additionally, the two LAMP primer sets $Nsp3_{-Park}$ and $Nos2_{-This-study}$ were (i) mapped on an alignment of 20 accessions, representing the 19 variants identified by GISAID to date and the GISAID hCoV-19 reference sequences, and (ii) blasted against the NCBI Refseq genome database for Coronaviridae (taxid : 11118), including SARS-CoV and MERS-CoV, with a total of 18,671 sequences.

Analytical sensitivity

Analytical sensitivity was evaluated using 10-fold serial dilutions ranging from 5,011,872.3 to 50.1 copies/mL (equivalent to 25,059.36 to 0.25 copies per reaction) of acid-heat inactivated SARS-CoV-2 isolate England/02/2020 (First WHO International Standard for SARS-CoV-2 RNA NIBSC code: 20/146), resuspended in negative saline swabs. Additionally, two positive clinical samples were included in the analysis. Prior to this evaluation, the 10-fold dilutions of the SARS-CoV-2 RNA NIBSC were tested with a RT-qPCR assay in order to generate a standard curve, by plotting the Ct values against the Log_{10} (copy number). RT-qPCR was performed as described in §2.1. SARS-CoV-2 genomic copy number in the two samples was then estimated by RT-qPCR. The two samples were then diluted in negative swabs to obtain serial dilutions from 200 to 0.8 copies/reaction and tested using the RUNCOV LAMP assay. The limit of detection (LOD) for which 100% of the replicates tested positive was determined for the standard and the two samples.

Validation using clinical samples

A first set of 46 RT-qPCR positive nasopharyngeal swabs was provided by the Biological Resource Centre (BRC) of the Centre Hospitalier Universitaire de la Réunion, Félix Guyon, Saint-Denis (hereafter, CHU). Samples collected from patients were placed in sterile tubes containing 1 mL PBS, and stored at the BRC at −80°C. Samples then were processed in parallel (i) on the one hand for RNA extraction (NucleoSpin® RNA Virus Kit, Macherey-Nagel) followed by RT-qPCR as described in §2.1 and (ii) on the other hand using the RUNCOV protocol as described in §2.3 on samples previously heated for 5 min at 95°C, followed by an additional 2-min heat step at 95°C on a 5 µL aliquot.

To assess the feasibility of conducting the test outside the laboratory setting, a second set of 294 clinical samples (110 RT-qPCR positive and 184 RT-qPCR negative), collected in physiological serum containing 1% inactivation solution, was tested on-site by the RUNCOV assay at a private medical biology laboratory (Cerballiance, France). Prior to triplex-RT-LAMP testing, all samples underwent heat inactivation for 5 min at 95°C, followed by an additional 2-min heat step at 95°C on a 5 µl aliquot. The results obtained from the RUNCOV system were then compared to RT-qPCR results obtained by the Cerballiance laboratory. RNA

extractions were performed from 300 μl swab samples using the Chemagic Viral DNA/RNA 300 kit H96 (Perkin Elmer) on the Automate Chemagic 360-D (Perkin Elmer) (elution in a final volume of 60 μl). The reactions were carried out on CFX96 Bio-Rad instrument using the SARS-CoV-2-RT-qPCR reagent kit (Perkin Elmer) (LOD = 20 copies/reaction for each viral target according to the supplier).

Statistical analysis

All statistical analyses were performed using the R statistical software (version 4.1.1 [2021-08-10]; R Development Core Team, 4, Vienna, Austria) with the packages nlme [22], lme4 [23], car [24], emmeans [25], tidyverse [26], and scales [27]. A linear mixed model was built to evaluate the effect of the medium (PBS, physiological serum or AMIES, fixed effect) on TTR variations, considering the sample as a random effect. A linear model was built to evaluate the effect of the protocol ("Control", "Trial 1", "Trial 2") on LAMP responses (positive, negative, inconclusive) and their interaction on TTR variations. Different variances were estimated according to the LAMP responses. A mean pairwise comparison test was used to compare least-squares means from the models. Wilcoxon signed rank tests on paired data were used to compare TTR values between simplex and triplex RNase P and between protocols (Trial 1, Trial 2, Control). A McNemar's Chi-squared test with continuity correction on paired data was used to compare the number of inconclusive results between the two treatment protocols (5 min at 95°C versus 5 + 2 min at 95°C). A pairwise Chi-squared test and a Fisher's exact test were used to compare the percentages of the inconclusive results among the RT-qPCR negative samples between Trial 1, Trial 2, and Control protocols.

Results

LAMP primers for human endogenous control

First, we selected the LAMP primer set initially designed by Curtis *et al.* [18], from the human *ribonuclease* (*RNase*) P reference sequence (GenBank accession number U94316.1). The choice was based on the compatibility between the Ta of the amplicon generated and the Ta of LAMP amplicons of the two candidate viral markers (see § 3.2). In the triplex LAMP assay, the concentration of the internal control LAMP primers had to be reduced to avoid potential competition between the human internal control and SARS-CoV-2 detection. The efficiency of the RNase P LAMP assay was first tested in a simplex assay to verify its efficiency at different primer concentrations on three negative samples (Supplementary Fig. S1). Consistent time-to-results (TTR) values (corresponding to the time values for which fluorescence takes off), were obtained for the different samples, even when the primer concentration was reduced to 0.25X, with mean TTR values ranging from 16.4 to 19.4 min (16-21 95%CI) (Supplementary Fig. S1). Concentrations higher than 0.25X showed an increased inhibitory effect on viral detection in preliminary results (data not shown). Conversely, the TTR values of the internal control obtained for the same three negative samples in the simplex RNase P 0.25X assay were not significantly different from the internal control TTR values obtained in the final RT-Triplex LAMP with RNase P primers 0.25X (Wilcoxon signed rank test, p = 0.6224). We also confirmed that the presence of the RNase P internal control primers did not affect the viral amplification, by comparing the viral TTR values obtained with RUNCOV on three positive samples, with the TTR values obtained for the viral duplex RT-LAMP (Pairwise comparisons using Wilcoxon signed rank test, p = 1).

Selection of LAMP primers and multiplexing optimization

The 11 LAMP primer sets were evaluated in a simplex format on a 10-fold dilution series of SARS-CoV-2 (Table 1 and Table S1). Most of the primer sets displayed good sensitivity with a LOD ("limit of detection", corresponding to the lowest viral dilution for which 100% of positive responses were obtained for the replicates) of 1/10,000 or 1/100,000 dilutions, corresponding to Ct values of 31.1 and 34.5, respectively (reference RT-qPCR, IP4 marker). Six primer sets had to be discarded for different reasons: i) Nsp3_1-61$_{\text{-Park}}$ and ii) Orf1ab$_{\text{-Zhang}}$ displayed a low sensitivity in the tested conditions, iii) Orf1ab$_{\text{-SONG}}$ system displayed low total fluorescence in several experiments compared to the other LAMP systems, iv) Spike$_{\text{-Park}}$ and v) Spir$_{\text{-This-study}}$ systems, which both target the spike gene, were considered as a mutational hotspot, as shown in the *in silico* study regarding the allele frequencies of mutations (Supplementary Fig. 1), and vi) N$_{\text{-Park}}$ due to several mutations at the 5′ end of the FIP primer in the Omicron variant. These mutations are predicted to impact the efficiency of the LAMP reaction [28].

Successful duplex format RT-LAMP systems (i.e. no inhibition between the two primer sets and sensitivity equivalent to simplex-LAMP assays) were obtained by combining LAMP primer sets with different melting temperatures (Ta values). For example, primer sets with high Ta like Nos2$_{\text{-This-study}}$ (88.4-88.5°C) were paired with systems with a lower Ta, such as Orf1a$_{\text{-Lamb}}$ (85°C), Orf8$_{\text{-This-study}}$ or Nsp3$_{\text{-Park}}$ (85.1-85.4°C) (data not shown). The primer set N$_{\text{-Zhang}}$ was discarded from further analysis since its Ta was incompatible with the design of a duplex LAMP with Orf1a$_{\text{-Lamb}}$, Orf8$_{\text{-This-study}}$ and Nsp3$_{\text{-Park}}$.

Lastly, three primer sets were selected and tested in a triplex RT-LAMP assay format with the RNase P as the internal control (Nos2$_{\text{-This-study}}$/RNase P paired with Orf1a$_{\text{-Lamb}}$, Nsp3$_{\text{-Park}}$ or Orf8$_{\text{-}}$

Table 2. Analytical specificity. Results of RUNCOV on non-target viruses.

Source	Virus	RUNCOV detection
CIRAD, UMR Astre, La Réunion	Influenza A (H1N1) pdm 09	0/2
CHU Saint-Denis, La Réunion	Human Coronavirus -1 229E	0/2
CHU Saint-Denis, La Réunion	Human Coronavirus -2 NL63	0/2
CHU Saint-Denis, La Réunion	Human Coronavirus -3 NL63	0/2
CHU Saint-Denis, La Réunion	Human Coronavirus -4 OC43	0/2
CHU Saint-Denis, La Réunion	Human Coronavirus -5 229E	0/2
Université de La Réunion, UMR PIMIT, La Réunion	Bat Alpha Coronavirus	0/2
ANSES Ploufragan, France	Pig Coronavirus (DEP)	0/2
ANSES Ploufragan, France	Poultry Coronavirus (BIV)	0/2

RNA extracted from different viruses were tested in two replicates. Tested viruses include animal viruses and respiratory viruses that cause clinical signs similar to those found during a SARS-CoV-2 infection.

$_{\text{This-study}}$) (Supplementary Table S2). Among the three systems, the triplex Orf1a$_{\text{-Lamb}}$/Nos2$_{\text{-This-study}}$/RNase P exhibited non-specific amplification in the NTC sample, with a Ta peak at 83.6°C, which indicates potential primer interactions (Supplementary Fig. S2). The other two systems (Nsp3$_{\text{-Park}}$/Nos2$_{\text{-This-study}}$/RNase P and Orf8$_{\text{-This-study}}$/Nos2$_{\text{-This-study}}$/RNase P) successfully amplified Omicron and Delta variants, showing annealing peaks for both markers. They also correctly amplified the internal control RNase P from the negative samples. The triplex Nsp3$_{\text{-Park}}$/Nos2$_{\text{-This-study}}$/RNase P was ultimately chosen for its location, as the Nsp3 region lies in the mainly conserved Orf1ab region of the genome, compared to the fast evolving Orf8 gene [29, 30] (see also Fig. 1C).

Optimizing the extraction-free preparation of the samples

First, we evaluated multiple swab collection media, PBS, AMIES/EswabR1 (Labellian, Nemours, France), UTM (Copan, Brescia, Italy), M4RT (Remel, Kansas, USA), using simplex or duplex format RT-LAMP assays on six clinical samples known to be positive for SARS-CoV-2. We observed a significant inhibition of the RT-LAMP reaction for samples collected in the UTM and REMEL media (data not shown). The mixed linear model demonstrated that the collection media (physiological serum, PBS or AMIES) had a significant effect on TTR from positive clinical samples (ANOVA, $p < 0.0001$). The average TTR values from samples diluted in physiological serum or PBS medium were significantly lower than samples diluted in AMIES medium (pairwise mean test, $p < 0.0001$ for AMIES versus the other two media, and $p = 0.9398$ for physiological serum versus PBS) (Fig. 2). Thirty clinical samples (11 RT-qPCR positive and 19 RT-qPCR negative samples) were then collected in physiological serum containing 1% inactivation solution and heated for 5 min at 95°C, as described by Rabe and Cepko (Rabe and Cepko, 2020), and tested by RUNCOV. All RT-qPCR positive samples tested positive with RUNCOV (Fig. 3, 11 first samples from the left, red dots). However, 12 qPCR negative samples (63.16%) displayed an inconclusive status. These samples did not generate a peak value or fluorescence signal for the RNase P control at $t \leq 25$ (Fig. 3, TTR symbolized by red dots at $t = 26$), except for one of the two replicates for each of the samples 10 and 20, which did not display a Ta peak, but had TTR values <25 (red dots surrounded by a dark circle). Also, contradictory results (und/neg) among replicates were also observed for the RT-qPCR negative samples 18 (Fig. 3). We optimized the picking-up of negative samples without compromising the detection of the positive samples, by incorporating an additional heat step of 2 min at 95°C in the sample preparation protocol. The modified protocol (5 + 2 min at 95°C) continued to show no false-negative results, but reduced the number of inconclusive status for RT-qPCR negative samples (Fig. 3, blue dots). Indeed, successful amplification of the RNase P control was observed for all of the RT-qPCR negative samples, except sample 6, which generated conflicting results (und/neg) between replicates. On whole data, TTR values were significantly earlier (Wilcoxon signed rank test, $p = 8.073e^{-11}$), and the proportion of inconclusive results was significantly lower (McNemar's Chi-squared test, $p = 0.0001768$) for the modified protocol (5 + 2 min at 95°C) than for the standard protocol (5 min at 95°C). Other unsuccessful strategies were tested to avoid this extra heating step (Supplementary data 1 and Supplementary Fig. S3); therefore, the modified 5 + 2 protocol was adopted for the extraction-free preparation of the samples.

Triplex RT-LAMP RUNCOV: workflow and examples

We developed an optimized workflow in 7 steps, by building on the multiple tests performed to define the primers, the optimal reaction conditions and steps in the RUNCOV protocol (Fig. 4). The samples are collected in physiological serum (or PBS) with 1% inactivation solution (step 1), and heated at 95°C for 5 min (step 2). Inactivated samples can be stored at room temperature for up to 90 min, otherwise at -80°C. Before the LAMP reaction five μL of each sample is heated at 95°C for 2 min in the microtubes (step 3), and then added to the reaction mix including the 18 LAMP primers (step 4). RT-LAMP is performed in a Genie II device, with amplification step at 65°C for 25 min, followed by a post-amplification step for 7 min (step 5). This RT-LAMP targets two regions of the SARS-CoV-2 genome, Orf1ab (Nsp3$_{\text{-Park}}$) and N (Nos2$_{\text{-This-study}}$) and an internal control, the human *RNase P* gene. For each sample, a fluorescence measurement is performed over time. A time to result value is generated, reflecting the amplification take off time during the amplification phase. During the post-amplification phase, annealing peaks are generated which are signatures of the amplified products (step 6). A sample is considered positive if (i) a TTR value ≤ 25 min is obtained and (ii) at least one of the specific annealing temperatures (Ta) for the viral targets is observed. For a negative test to be deemed valid, the internal control must show an amplification peak within the specific annealing peak. If no amplification is detected for internal control or for SARS-CoV-2, the test is considered inconclusive. (step 7). The total run time from sample collection to final result is typically 40 min. Examples of amplification curves, annealing peaks and automatic readouts are shown in Fig. 5.

It is worth mentioning that the annealing temperature can vary depending on the medium used for collecting samples. The range provided here is suitable for RNA extracts or samples collected in physiological serum or PBS, supplemented with an inactivation solution.

RUNCOV performance evaluation

Analytical specificity

No false-positives were obtained when RUNCOV was used on non-target viruses, including other common coronaviruses of human origin (Table 2).

High specificity was also supported by *in silico* genome sequence analyses. In the 13,538,889 unique genome sequences of SARS-CoV-2 available, 97.9% show 100% identity with priming sites of at least one system. By allowing mismatches that have no impact on LAMP efficiency in the regions targeted by the primers (internal region and 5' terminal region for F2, B2, F3, B3 and internal region and 3' terminal region for F1c, B1c) [28], the percentage increased to 99.25%. The 19 accessions representing the 19 GISAID variants corresponded to 12 sequence types; some accessions had identical sequences at the loci corresponding to the Nsp3$_{\text{-Park}}$ and Nos2$_{\text{-This-study}}$ LAMP primer sets (Supplementary Fig. S4). When mapping the Nsp3$_{\text{-Park}}$ primer set on the alignment, only one of the eight primers (LF) presented a mismatch, for the GISAID 17 variant, which was located in the central part of the primer and does not theoretically impact the system's performance. Mapping the Nos2$_{\text{-This-study}}$ primer set on the alignment showed that no mismatch was identified for four of the eight primers (F1c, B1c, LB and B3). Mismatches were located in the central zone for three of the four other primers (F2, LF and B2), with no theoretical impact on performance [28]. The remaining mismatches observed in three sequences within the F3

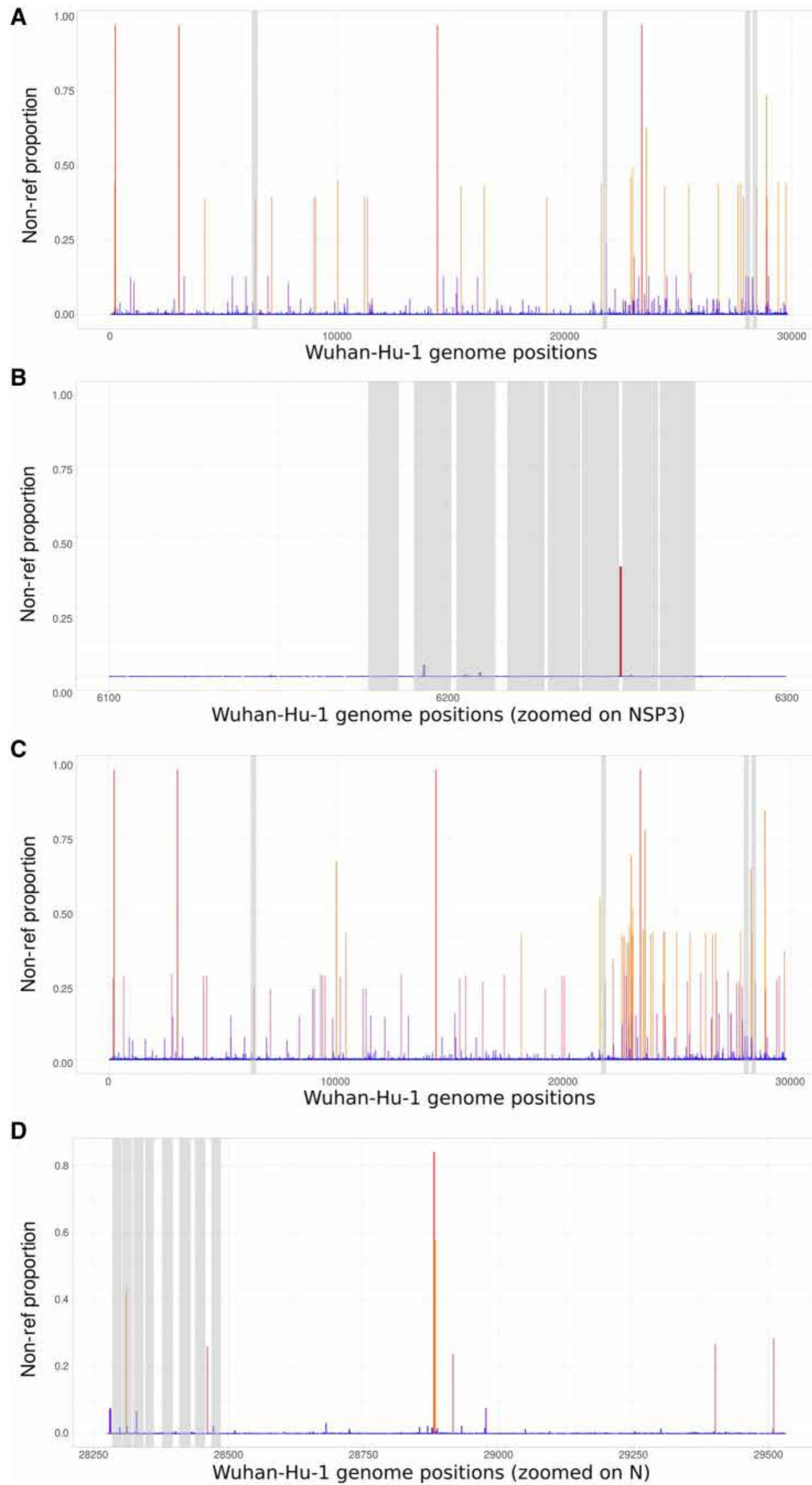

Figure 1. Frequencies of non-reference alleles along the SARS-Co-V2 genome in the global population. (A): Non-reference allele frequencies of a dataset dating from the early phase of this study, comprising 207,435 genomes randomly subsampled in January 2022 from the Gisaid database. Grey bars represent candidate target regions, from left to right: Nsp3$_{\text{-Park}}$, both Spike$_{\text{-Park}}$ and Spir$_{\text{-This-study}}$ systems, Orf8$_{\text{-This-study}}$, and Nos2$_{\text{-This-study}}$. (B): zoom on the genome region targeted by Nsp3$_{\text{-Park}}$ primers. (C): same as (A), using a dataset dating from the final phase of the study (September 2023, 400,829 genomes); same legend for the grey bars as in (A). (D): zoom on the genome region targeted by Nos2$_{\text{-This-study}}$ primers

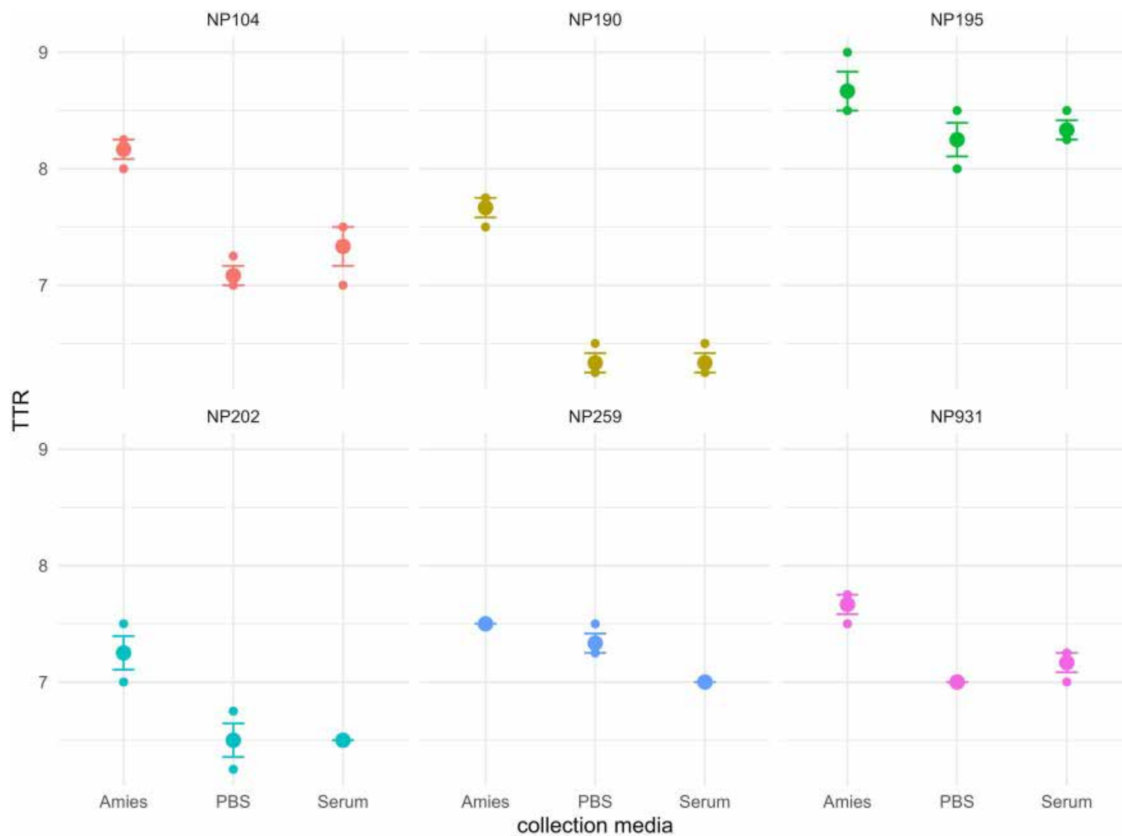

Figure 2. TTR values (Min) obtained from RUNCOV on six positive clinical samples (NP104, NP190, NP195, NP202, NP259, NP931, with a distinct colour for each sample) diluted in either AMIES, PBS or Serum. Observed values (triplicates) are represented by small pale dots, mean values by dark dots, and 95% confidence intervals are shown by bars

primer involved GT pairings. These are known to have no discernible effect on the efficiency of molecular amplification, even when situated in the 3' end of the primer, due to sufficiently strong bonds [31]. Lastly, the $Nsp3_{-Park}$ LAMP primer set did not show significant similarity to any Coronaviridae other than the SARS-CoV-2 genome sequences, including SARS-CoV and MERS-CoV, in the NCBI Refseq genome database during *in silico* analysis. Using the same database the $Nos2_{-This\text{-}study}$ primer set only matched with the SARS coronavirus Tor2 genome sequence (NCBI: txid227984) with a low percentage of identity: 85.99%. The mapping of the $Nos2_{-This\text{-}study}$ primer set on the Tor2 genome sequence exhibited redhibitory mismatches that could compromise LAMP efficiency, notably five successive SNP mutations at the 5' end of the B1c segment of the FIP primer, and a SNP mutation at the 3' end of the F2 region (Supplementary Fig. S5) [32].

Analytical sensitivity (ASE)

Dilution of SARS-CoV-2 NIBSC reference 20/146 in negative swabs

When tested on 10-fold dilutions (5,011,872.3 to 50.1 copies/mL, i. e. 25,059.36 to 0.25 copies/reaction) of the inactivated virus in negative swabs, RUNCOV displayed a very high sensitivity level of 100% (6 out of 6 replicates) at the concentration of 25 copies per reaction (Fig. 6). The corresponding Ct values obtained by RT-qPCR (mean ± sd) were 31.21 ± 0.14 and 33.06 ± 0.1 for IP4 and IP2, respectively.

Serial dilutions of clinical Covid-19 samples

The sensitivity was evaluated using two clinical nasopharyngeal samples tested positive for the Delta (sample NP215) and Beta variants (sample NP194) that were diluted in negative swab samples (Fig. 6). The viral concentration of each sample was estimated using real-time RT-qPCR. Ct values of 17.05 and 14.16 were obtained for NP215 and NP194, respectively, corresponding to concentrations of 768,148,441 and 5,802,267 copies/reaction when reported to the standard curve obtained with the real-time assay performed on the serial dilutions in negative reference swabs of SARS-CoV-2 NIBSC reference (Ct= −3,291 × Log_{10} (Conc) + 36.419, efficiency = 101.3%, r^2 =0.999). The LOD values obtained with the RT-LAMP assay were 20 and 5 copies/reaction for NP215 and NP194, respectively. The corresponding experimental Ct values measured at these dilutions were 30.77 ± 0.05 (IP4) and 32.26 ± 0.08 (IP2) for NP215, and 31.70 ± 0.08 (IP4) and 33.38 ± 0.11 (IP2) for NP194. These results are consistent with previous results obtained with the NIBSC reference and are within the same range.

Diagnostic specificity and sensitivity

The triplex RT-LAMP performance was assayed on 340 clinical samples composed of a first set of 46 originating from CHU and a second set of 284 originating from Cerballiance, all compared to the results of the RT-qPCR. Among these clinical samples, 156 tested positive and 184 tested negative with the RT-qPCR.

Among the 156 RT-qPCR-positive samples, only four samples tested negative with the triplex RT-LAMP, two samples from Cerballiance, displaying Ct values of 32 and 33, and two samples from the CHU with Ct values of 34 and 35.6 (Fig. 7). It is worth noting that the TTR values of the CHU samples were generally higher compared to Cerballiance. This is probably because the CHU samples were collected earlier in the project and underwent

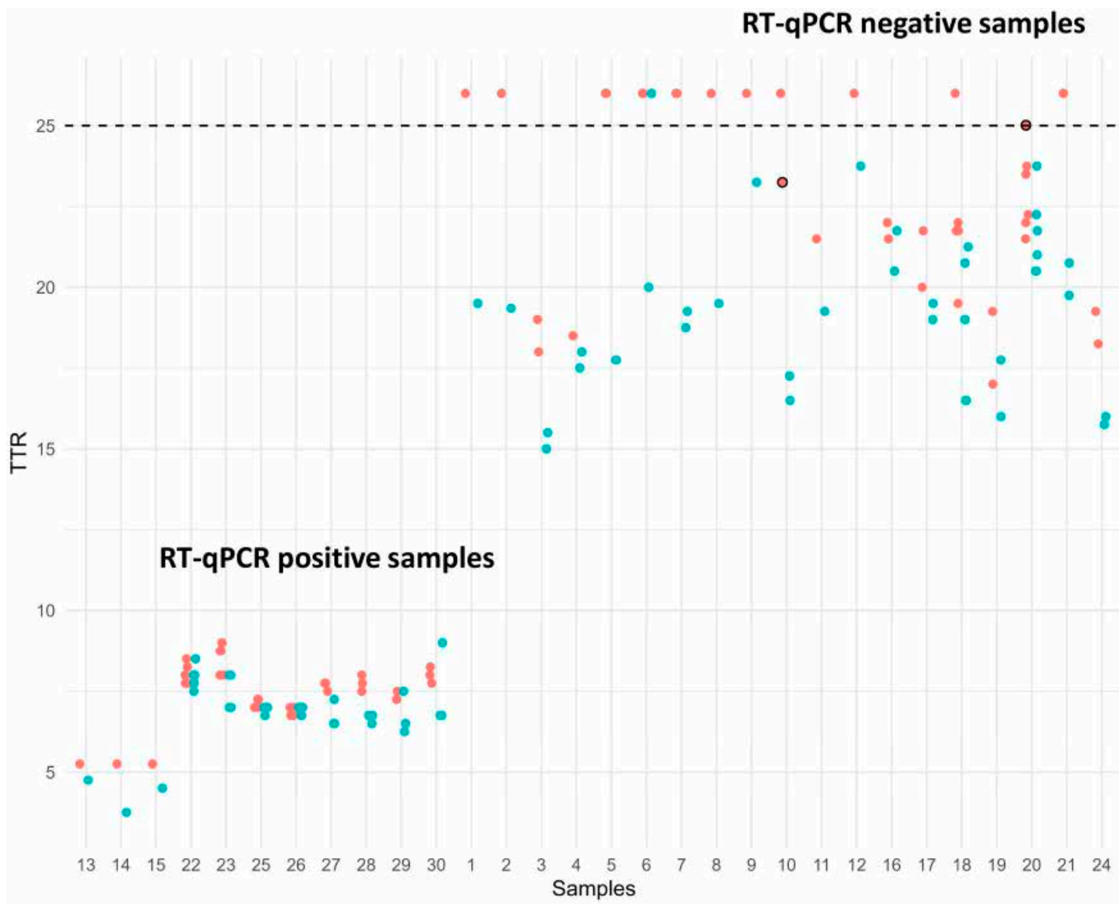

Figure 3. RUNCOV TTR values (Min) obtained for 11 RT-qPCR positive samples (left) and 19 RT-qPCR negative samples (TTR obtained for RNase P control) (right). Samples were previously heated at 95°C for 5 min in the collection tubes (red dots) or at 95°C for 5 min plus additional heat of 2 min at 95°C in LAMP microtubes (blue dots). The qPCR negative samples displaying neither a LAMP fluorescent signal nor Ta peak (inconclusive) are symbolized by a red dot above the limit of detection, at t = 26. The two red dots surrounded by a dark circle are also inconclusive results with no Ta peak, but with a TTR value <25. The dotted bar represents the limit of TTR detection (25 min)

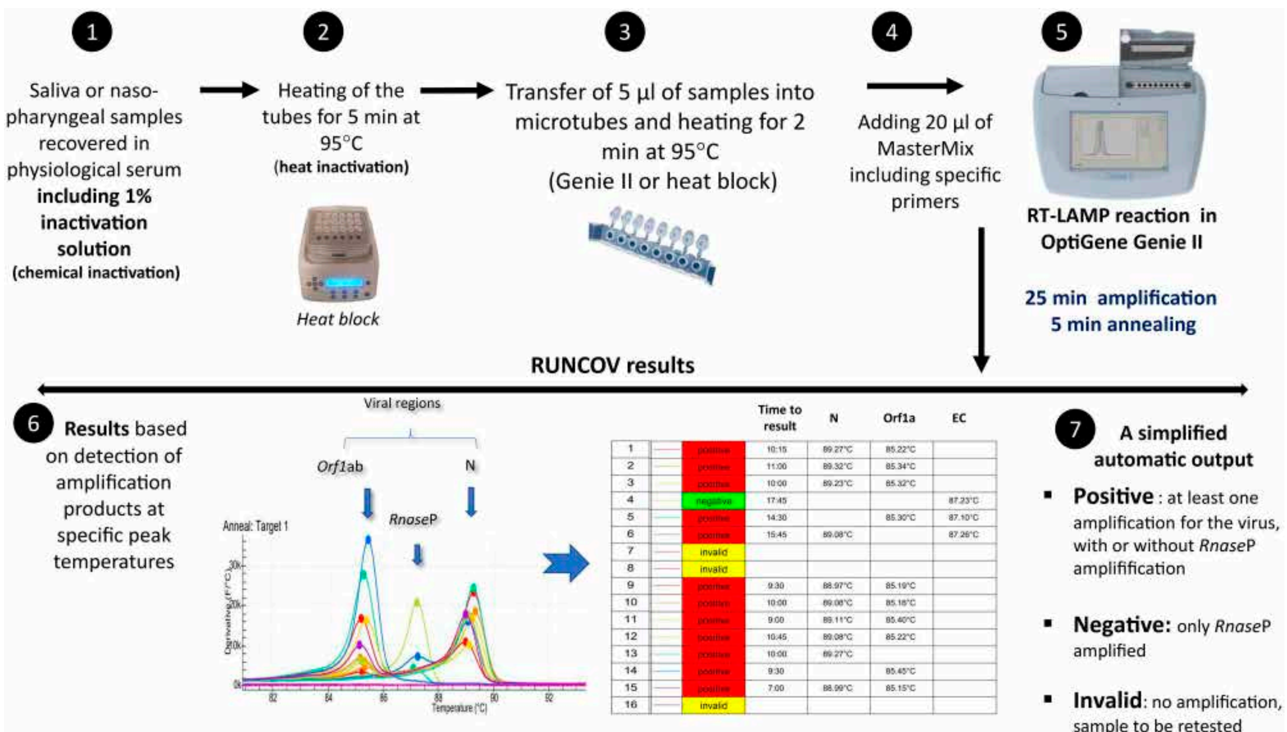

Figure 4. RUNCOV workflow

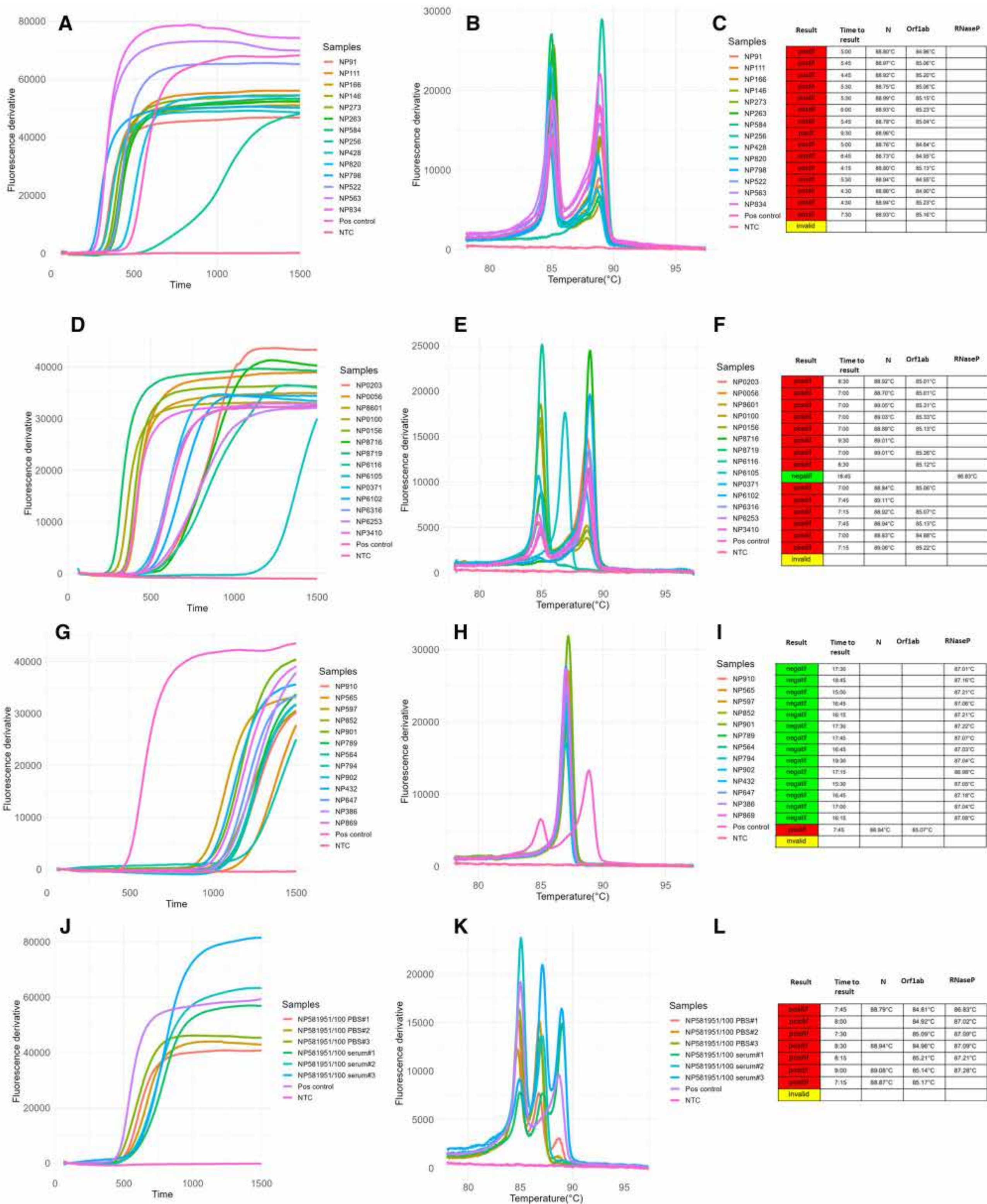

Figure 5. Amplification plots (left), annealing peaks (medium) and automatic readouts (right) obtained for clinical samples tested with RUNCOV. (A), (B) and (C): positive samples. (D), (E) and (F): positive samples with one negative sample. (G), (H) and (I): negative samples. (J), (K) and (L): low positive samples, both viral and endogenous control Ta peaks are present

at least one thawing/refreezing cycle before the final test, two years later.

Among the 184 RT-qPCR negative samples, a total of 179 samples were amplified with the internal control RNase P, resulting in an inconclusive rate of 2.72% among the RT-qPCR-negative samples, and a percentage of 1.47% when considering all samples in the trial. Upon re-testing, only one sample remained inconclusive, lowering the rate of inconclusive results to 0.54% for the RT-qPCR-negative samples and 0.29% considering all samples.

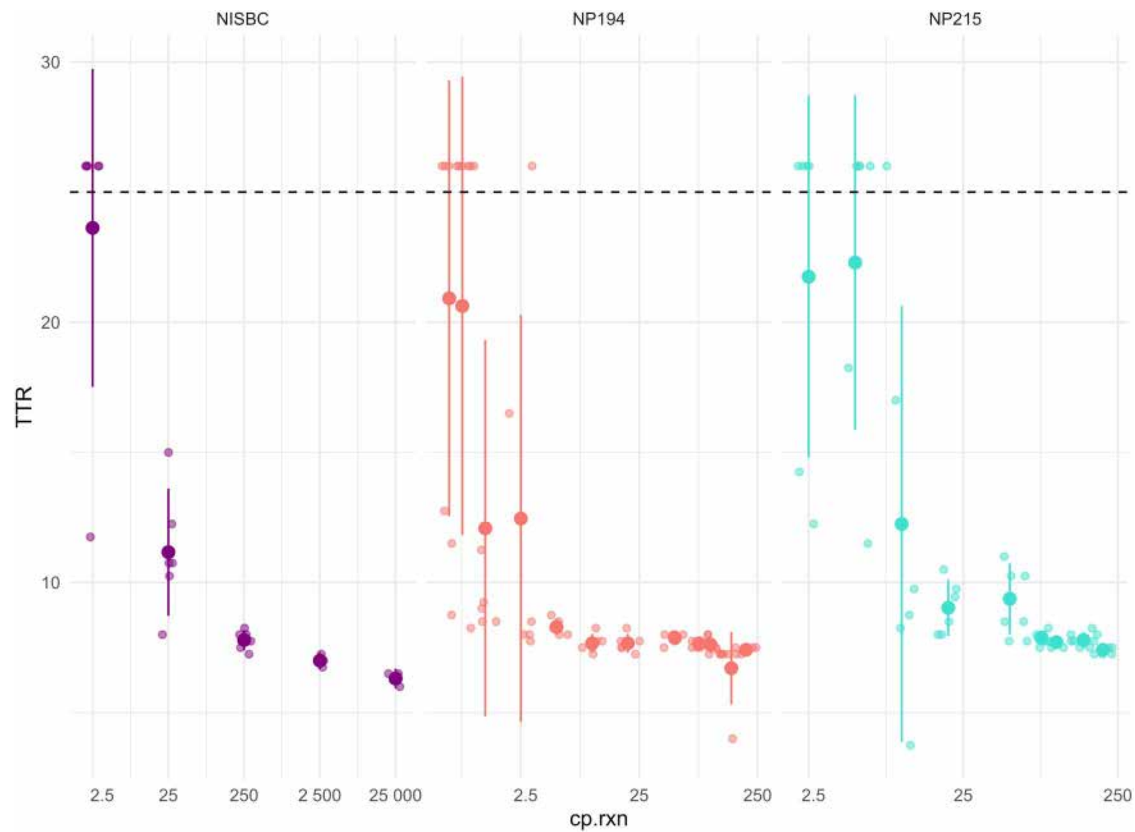

Figure 6. RUNCOV detection on serial virus dilutions of SARS-CoV-2 in negative swabs. left: NIBSC reference 20/146. center and right: clinical nasopharyngeal positive samples NP194 and NP215, respectively. Six replicates per dilution level and per sample; cp/rxn=copies/reaction. Replicates are represented with small pale dots, mean values with dark dots and 95% confidence intervals with bars. The dotted bar represents the limit of TTR detection (25 min)

Table 3 summarizes the diagnostic specificity (Dsp) and sensitivity (Dse) values obtained for the clinical data from Cerballiance and all combined clinical data. The sample that was detected as inconclusive twice was excluded from the calculation of diagnostic metrics but was mentioned in the contingency table, as recommended by Shinkins *et al.* [33].

For the Cerballiance clinical data, Dse = 98.18% (95% C.I [93.59% to 99.78%]), Dsp = 100% (95% CI [98.00% to 100.00%]), the positive predictive value (PPV) is 100% (95% CI [94.64% to 100%]), the negative predictive value (NPV) is 98.92% (95% CI [95.86% to 99.72%]), the accuracy is 99.32% (95% CI [97.56% to 99.92%]), the positive likelihood ratio (PLR) is ∞ (no false positive), and the negative likelihood ratio (NLR) is 0.02 (95% CI [0.00–0.07]). This indicates almost perfect agreement between the two assays. When combined with the CHU samples, Dse = 97.44% (95% C.I [93.57% to 99.30%]), Dsp = 100% (95% C.I [98.00% to 100.00%]), PPV= 100% (95% CI [97.60% to 100.00%]), NPV= 97.86% (95% CI [94.56% to 99.18%], the accuracy is 98.82% (95% CI [97.01% to 99.68%], PLR= ∞ (no false positive) and NLR = 0.03 (95% CI [0.00–0.07]). This again indicates an almost perfect agreement between RT-LAMP and RT-qPCR.

Discussion

We have developed a sensitive and reliable diagnostic tool capable of detecting all variants of SARS-CoV-2 known to date. It can be used directly on clinical samples and does not require RNA extraction. This alternative molecular test for SARS-CoV-2 can easily be deployed for population screening, particularly when access to standard RT-qPCR-based approaches is limited.

The appeal of this rapid and sensitive molecular tool is strengthened by its ease of use. It requires very few handling steps and includes a process control in the absence of a virus. Moreover, its low cost makes it competitive, since it does not require a complex extraction process or an additional revelation step using probes.

Developing this high-performance tool involved several challenges, e.g. the need to create a sensitive molecular tool for virus detection that is capable of producing a negative result in the absence of the virus. To overcome this, we developed a triplex RT-LAMP, amplifying two viral genome regions and an endogenous human control.

Several criteria were considered when selecting the viral targets: (i) LAMP primers had to be chosen in different parts of the genome, by considering the balance between the stability and specificity of the target regions. The N and Orf1ab regions were selected as they are specific to SARS-CoV-2 and do not include mutational hot spots, as revealed by the extensive *in silico* analysis, performed in 2022 and updated in 2023; (ii) Each amplicon's Ta had to be distinct to create unique signatures for the amplified regions of the genome; (iii) Experimental validation was performed to ensure the compatibility of different primer sets and the absence of primer cross-dimers in order to avoid negative interactions between LAMP primers.

A similar approach was also adopted for the addition of an internal human control. The role of this control is important because it confirms that the absence of viral responses is not due to

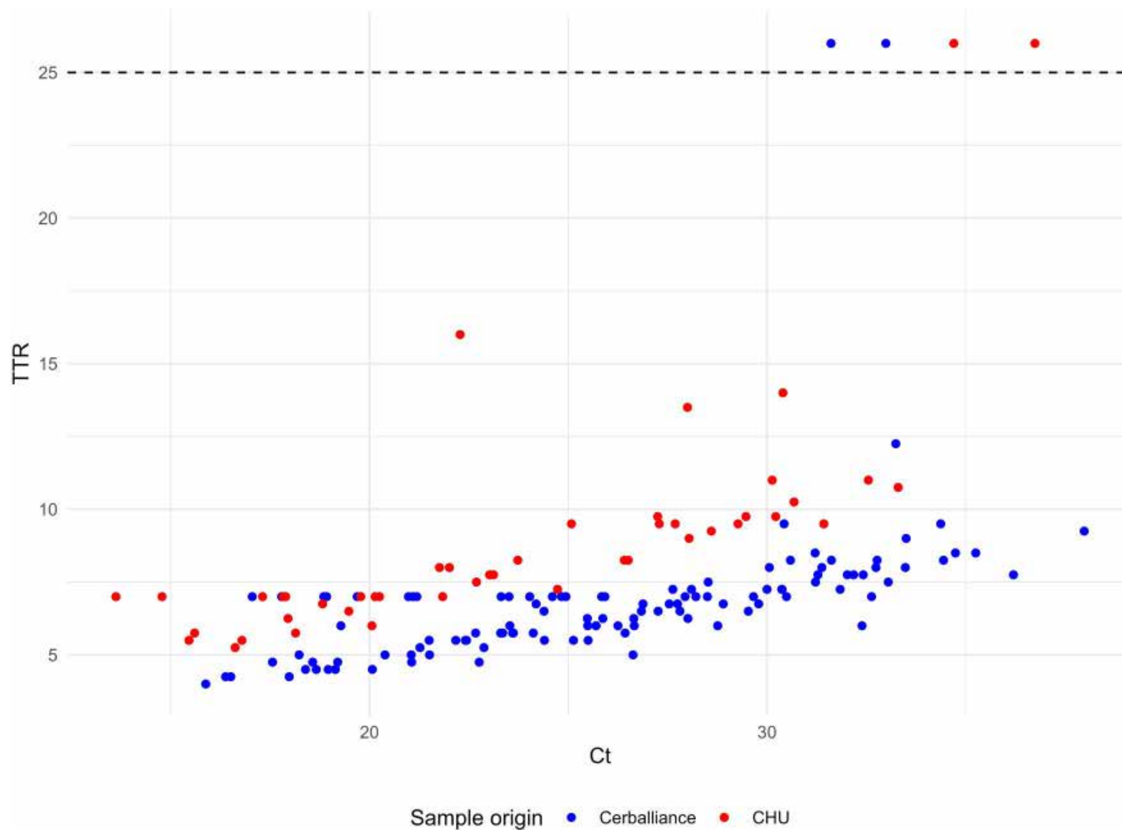

Figure 7. Triplex RT-LAMP time to results (TTR) in minutes of individual samples plotted against RTq-PCR Ct values. Red dots represent 112 SARS-CoV-2 positive (CT ≤ 40) clinical samples, as determined by RT-qPCR tested at the Cerballiance laboratory's drive station. Blue dots represent 44 SARS-CoV-2 positive (CT ≤ 40) clinical samples, as determined by RT-qPCR collected at CHU La Réunion and sent to CIRAD for analysis. The dotted bar represents the limit of TTR detection (25 min)

a failure in any step of the protocol, i.e. sampling, RNA preparation and/or LAMP amplification. A LAMP primer set targeting the human *RNase P* gene, with a Ta compatible with those of the viral LAMP sets successfully yielded an amplification from the human *RNase P* gene when no viral target was amplified. This LAMP primer set has already been used successfully as an internal control for SARS-CoV-2 negative nasopharyngeal swabs and other human viruses, as described in several publications [18, 34]. In the context of LAMP reactions, internal controls can hinder the successful detection of the target [35]. This inhibition can be due to the depletion of limiting reagents in the reaction mix or to interactions between the different primers used. Therefore, the performance of the internal control was intentionally impaired, by decreasing the concentration of the RNase P primer set to 0.25X of the standard concentration, as described by Zhang and Tanner [36]. Under these conditions, only 1.47% of inconclusive responses were obtained for the samples in the clinical trial. Comparing these results with probe-based RT-LAMP multiplex assays that include an internal control is challenging, as reported rates for inconclusive results vary widely (ranging from 0% to 46%) [35–37]. Nonetheless, comparable rates of 1.2% [38] and lower rates of 0.5%-0.9% [39] have been reported for qPCR assays. Interestingly, a higher rate of 3.6% was observed for a qPCR assay conducted without sample extraction [38, 40]. The primary reason for inconclusive results in LAMP and other molecular methods directly applied to samples is likely the presence of high concentrations of polymerase inhibitors in the samples. Another potential contributing factor could be unreliable sampling. These inconclusive results typically necessitate re-testing. In our study,

Table 3. Diagnostic specificity and sensitivity.

A		RT-qPCR Positive	RT-qPCR Negative
Direct triplex RT-LAMP	Positive	108	0
	Negative	2	183
	Inconclusive	0	1
	Total	110	184

Dse = 98.18%, 95% C.I. = [93.59% to 99.78%],
Dsp = 100%, IC 95% [98.00% to 100.00%]

B		RT-qPCR Positive	RT-qPCR Negative
Direct triplex RT-LAMP	Positive	152	0
	Negative	4	183
	Inconclusive	0	1
	Total	156	183

Dse = 97.44%, 95% C.I [93.57% to 99.30%],
Dsp =100%, IC 95% C.I [98.00% to 100.00%]

re-testing reduced the percentage of inconclusive results to a very low percentage of 0.29%.

Another crucial aspect of this study involved the preparation of the sample before conducting LAMP testing. Since this POC tool is designed for use outside a traditional laboratory setting, we focused on developing a rapid and simplified method for sample preparation. Specifically, we aimed to enable direct preparation from nasopharyngeal swab collection media. Prior research

indicated that conducting a direct LAMP reaction from swabs could be hindered by the presence of certain additives in classical viral transport media (VTM), such as buffered agents and compounds that inhibit the growth of bacteria, fungi and other microorganisms, as observed in universal transport medium (UTM) or some VTM. In contrast, simple media like physiological serum and phosphate-buffered saline (PBS) were found to be suitable for LAMP reaction according to various publications [41–43]. Our own findings confirmed these data.

The saline solution collection tubes with 1% of inactivation solution (containing TCEP and EDTA) were heated at 95°C, as recommended by Rabe and Cepko [44]. This quick step inactivates the virions and also protects the viral RNA from RNases. Indeed, TCEP is capable of reducing any disulfide bridges within and between proteins and, thus, is involved in protein denaturation. Additionally, EDTA can chelate the divalent cations necessary for RNase activity. However, an additional step of heating for 2 min at 95°C in the microtubes was required to detect the human internal control and prevent samples from being labelled as inconclusive. The extra 2-min heating step in a small volume (5 μL sample in 100 μL microtube), probably results in the faster and more efficient release of nucleic acids and the neutralization of potential amplification inhibitors, compared to simply heating the initial 1 mL sample in a 2 mL collection tube. Although satisfactory results were achieved by heating 5 μL native samples directly for 5 min at 95°C in the LAMP microtubes containing lyophilized inactivation solution at the bottom (data not shown), this approach was not used in our study in order to protect operators from potential exposure to the native virus.

In these improved experimental conditions, RUNCOV was able to detect the wild type and different variants of concern, such as Beta, Delta and Omicron. This was supported by an extensive *in silico* analysis based on 13,538,889 non-redundant genomic sequences, which revealed that 97.9% of SARS-CoV-2 genomes had 100% identity to priming sites of at least one targeted region. The figure was 99.25% when mismatches of no consequence to LAMP efficiency were allowed. Indeed, the chosen targets demonstrated relative stability over time, with the appearance of only a few mutations. Additionally, dsDNA binding dye-based RT-LAMP has been shown to be tolerant to mutation points, with very little impact in terms of single-base changes in SARS-CoV-2 mutations, compared to RT-qPCR assays or beacon probe-based-RT-LAMP [45]. The tolerance of RT-LAMP to sequence variation is a significant advantage compared to other methods. On the other hand, the perfect analytical specificity of RUNCOV was evidenced when testing on non-target isolates. This result was supported by a thorough *in silico* analysis performed on non-target genomes, including genetically closely related non-target genomes. Interestingly, given the careful selection of primer sets, false-positive results due to primer dimers were never observed, despite the presence of a high number of primers (18) in the mix. Another advantage of Ta-based RT-LAMP over other methods, such as colorimetric methods, is the ability to check the Ta of amplicons that corresponds to the observed amplification signal, which means primer dimers can easily be distinguished from expected amplicons.

The analytical sensitivity of RUNCOV ranges between 5 and 25 copies per reaction, assessed using both the international standard for SARS-CoV-2 RNA and clinical samples diluted in negative swabs. The slight variations in sensitivity may be attributed to differences in the composition of various human negative swabs. LAMP is known to be more tolerant to inhibitory compounds than PCR [46, 47], although its sensitivity may be affected by the nature and quantity of biological substances [46, 48].

This analytical sensitivity value is slightly lower compared to an optimized RT-qPCR assay, which has a theoretical LOD of three copies per reaction, when only the Poisson distribution contributes to replicate variation [49, 50]. However, this limit of detection is equal or even better than the values observed for other specific COVID-19 RT-LAMP assays [51–53]. Interestingly, negative results for LAMP in the clinical trial were seen at Ct values of 31 and above, similar to those found during the analytical sensitivity analysis. Nevertheless, RUNCOV was able to detect higher Ct values. Very high diagnostic sensitivity and specificity values were obtained with overall rates of 97.37%, 95% C.I [93.40% to 99.28%] for sensitivity and 100%, 95% C.I [98.02% to 100.00%] for specificity. Pu *et al.* [54] conducted a meta-analysis on 18 RT-LAMP tests and showed that the pooled sensitivity and specificity of RT-LAMP for diagnosing COVID-19 were 0.92 (95% CI, 0.85 – 0.96) and 0.99 (95% CI, 0.99 – 0.99), respectively. Nevertheless, all these RT-LAMP assays were performed using RNA extracts and diagnostic sensitivity values of direct RT-LAMP are usually much lower [41, 52, 55].

Conclusion

We have developed a rapid, highly sensitive, and specific RT-LAMP assay capable of simultaneously detecting two different genes (Orf1ab and N) of SARS-CoV-2, as well as an internal control, following a very simple sample preparation procedure. RUNCOV exhibited high diagnostic sensitivity (97.4.5%) and specificity (100%) compared to the RT-qPCR gold standard. The tool's proven portability is another original feature. Indeed, it has already been successfully implemented in various non-laboratory settings in La Réunion. Although a large number of RT-LAMP kits have been published and commercial kits made available, none appear to combine all the features described for RUNCOV in this manuscript: simplicity, no prior RNA purification, affordability, good specificity and sensitivity, and multi-targeting capabilities including an internal control. This test evaluated in 2020 by the French National Reference Center for Respiratory Diseases ranks as one of the five RT-LAMP COVID-19 tests authorized in France during the COVID-19 pandemic. Although the COVID-19 vaccines have effectively prevented SARS-CoV-2 infection and reduced the number of COVID-19-related deaths [56], some health and/or age-related conditions are still associated with a higher risk of severe illness [57, 58] or the development of post-COVID syndrome [59]. Our quick and reliable detection method will benefit patients, particularly those in vulnerable groups, and has the potential to prevent severe outcomes.

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Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

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Ethical statement

All samples were collected after individual notification, with no opposition from the persons involved, as part of the sample requalification research protocol pertaining to the COVID-EPI protocol used by the CHU in la Réunion. The protocol was approved by the infectiology ethics committee (CER-MIT) under N°2020-0403. Residual samples were also used from viral diagnostic tests in the framework of the collaborative contract between CIRAD and Cerballiance. No data on age, sex, or ethnic origin were collected. The studies were conducted in accordance with CNIL reference methodology MR-004 (declaration of conformity numbers 221343) and in compliance with current General Data Protection Regulation requirements.

Conflict of Interest

The authors declare no conflict of interest.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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