Rapid, multiplex detection of SARS-CoV-2 using isothermal amplification coupled with CRISPR-Cas12a

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Article

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Abstract

In December 2019 an outbreak erupted due to the beta coronavirus severe acute respiratory syndrome (SARS)-CoV-2 in Wuhan, China. The disease caused by this virus (COVID-19) rapidly spread to all parts of the globe leading to a global pandemic. Efforts to combat the pan-demic rely on RT-qPCR diagnostic tests that have high turnaround times (~24h), are easily contaminated, need specialized equipment, facilities, and personnel that end up increasing the overall costs of this method. Loop-mediated isothermal amplification (LAMP) coupled with a reverse transcription step (RT-LAMP) is an alternative diagnostic method that can easily overcome these obstacles, when coupled with CRISPR/Cas it can eliminate false positives. Here we report a fast (~40 min), highly sensitive, point-of-care RT-LAMP and CRISPR/Cas12a assay to detect SARS-CoV-2. This fluorescence-based test prevents carry-over contamination using dUTP/UDG and achieved 100% specificity and 93% sensitivity using 25 positives and 50 negative patient samples for Ct<35. Our reported LoD of 3 copies/µL will enable the robust, fast detection of the virus in a dedicated equipment which is a major step towards population-wide accessible testing.

Introduction

The 2019 outbreak of SARS-CoV-2 that began in Wuhan China and led to a global pandemic caused a massive overload of health systems. The disease called COVID-19 rapidly spread to the entire globe, being transmitted by droplets that contain high viral loads capable of infecting an individual. As of 21 of September 2021, there are a total of 228 million infections worldwide with a death toll of 4.7 million people (WHO).

For the diagnostic of viral agents, nucleic acid amplification tests (NAAT) are the most used diagnostic tool and have been extensively reported during past outbreaks of different coronavirus such as MERS and SARS-CoV. The current “gold standard” to detect active SARS-CoV-2 infections is RT-qPCR. This technique has a high efficiency while generating a large number of amplicons (~ 109) [1] [2]

In a low-resource environment these types of tests are inaccessible, there is thus a need to develop affordable and point-of-care diagnostic tools to provide low- and mid- income countries with the necessary tools to accurately track infections. To remove the need of using a thermocycler, isothermal nucleic acid amplification methods were the first choice, the constant temperature of the reaction and short amplification times allow for the miniaturization of the equipment and a more point-of-care approach [3], [4]

LAMP (Loop-mediated isothermal amplification) attracted more attention. This technique can be coupled to a reverse transcription step with colorimetric, turbidity and/or fluorescence-based detection of amplicons [5]–[7]. It is conducted between 60–65ºC and uses 4 to 6 primers (PCR only uses 2) complementary to different regions within the analysed DNA in combination with different DNA polymerases that have strand displacement activity. Multiple tests have been developed using this method, to detect infectious agents such as Zika, influenza, tuberculosis, and malaria [7]–[9]. Despite

being an extremely fast amplification method (around 30 minutes), or even due to this, these methods are associated with a high propensity to generate false positive results. To tackle this problem, new diagnostic technologies relying on CRISPR/Cas are being developed [10][11][12][13]

To tackle the false positive problem several innovative solutions have been developed mainly using RNA-guided CRISPR/Cas detection of nucleic acids since it allows for high sensitivity, specificity, and reliability. The basis of this technology is using a Cas nuclease associated with a specific guide RNA (gRNA) that will determine the target sequence of the effector protein. Once this protein is activated (target sequence is present) it will cleave surrounding single-strand nucleic acids indiscriminately (collateral cleavage) due to its intrinsic trans nuclease activity. This activity can enable nucleic acid detection by using guide sequences specific for the product of the isothermal amplification, leading to the cleavage of an F-Q reporter enabling fluorescence-based detection of the viral genetic material [10], [14], [15][16].

Results

Detection of SARS-CoV-2 by RT-LAMP

Commercially available open-source kits.

It is possible to achieve very low sensitivity by combining isothermal amplification methods (RT-LAMP) with the simultaneous detection of different target sites on the SARS-CoV-2 genome by the CRISPR/Cas12 system. When detecting SARS-CoV-2 via RT-LAMP, we found that commercially available kits could only achieve a significant response to 30 copies/µL, high propensity to produce false positive results and recurrent on/off behaviour at lower or higher copy numbers. These kits did not achieve the desired limit of detection nor the desired specificity for the project at hand (Fig. 1, Fig. 2).

Both kits showed positive amplification at 10 min for high viral loads and positive controls, despite this, the sensitivity of the assay was not very good and did not allow for the robust and consistent detection of lower viral loads indicating that the amplification was still producing low amounts of amplified material and thus, there was room for improvement.

Using a combination of the primer sets supplied with these kits it was possible to further increase the limit of detection of the system to 10 cp/µl of the stock solution. This multiplex approach in-creased the consistency of the results but still showed non-specific amplification within the desired reaction time. Several reports showed that GndHCL and L-pro could be promising enhancers of both the RT-LAMP reaction and the Cas12a detection, we decided to investigate these compounds [17], [18].

To increase the limit of detection, different additives were investigated, namely Guanidine hydrochloride (GndHCL) which has demonstrated the ability to increase the speed of the reaction as well as the specificity of the interaction between primers and their specific targets (Fig. 2a). Supplementing this reaction with more Bst 2.0 polymerase (final concentration of 0,32U/µl) on top of the one present in the

master mix (undisclosed amount) greatly increased the speed of the re-action and even showed amplification at 1 copy/µL. These conditions reduced the start of amplification for higher viral load samples to 15 min while reducing the limit of detection to 1 copy/µl of stock solution and thus 0.2 copies in solution (Fig. 2b)

CRISPR/Cas12a based detection of synthetic RNA controls

When using the less optimized reaction as input to the Cas12a incubation step, the end-point signal produced was very low (Fig. 3a) and no saturation was observed (Fig. 3b). This agreed with the results obtained previously. Nonetheless, to maximize the end point signal some adjustments were made in the Cas12a reaction, namely, reducing incubation from 30 to 20 min; using diluent A to dilute the enzyme, adding the FAM-BHQ substrate after the incubation step and using 2µl of input RT-LAMP reaction as it produces the best signal-to-noise ratio.

After all the kit components were defined, the amplification of individual genes was once again tested (Fig. 3c) showing almost 10-fold increase in the end point signal for the multiplex reaction and 2-fold higher for the individual reactions and an almost sigmoidal pattern for the enzymatic reaction (Fig. 3d).

While the amplification of an individual gene (E) can yield a moderate positive signal at high viral loads, the combination of the multiple components makes the reaction more robust and with a final signal 5X higher than the original reaction (Fig. 3e). It was possible to observe a plateau for the enzyme kinetics (saturation) and increased reaction speed. This combination of multiple guide RNAs achieved lower or similar detection limits to tests that are currently on the market and thus can be a promising method to achieve cheap, point-of-care testing for the current and future outbreaks.

Melting curve analysis of amplified products

To verify that the amplification assay was indeed successful, we evaluated the fluorescence of the intercalating dye via melting curves. As the temperature increases, the double strand of DNA produced via RT-LAMP was denatured and split into ssDNA Since we used an intercalating dye, as soon as dsDNA turns into ssDNA the fluorescence intensity dropped and could estimate of the strength of the hydrogen bonds of the fragment produced which is specific to the virus. From the obtained melting curves, we observed that the N gene presented non-specific amplification in the melting curve indicating a contamination of the non-template control wells (Fig. 4a). This contamination is always a concern with these isothermal amplification methods since there is exponential amplification rather than cycle based. Both individual genes, E gene in Fig. 4b and Orf1a in Fig. 4c, and multiplex (Fig. 4d) were amplified with specific melting points and without non-specific amplification. Clearly, lower copy amounts produced less amplicons for all the genes. In the multiplex situation it might be easier to detect lower copy amounts since there is a simultaneous amplification of different genes which can produce a favourable fluorescence signal when detected by Cas12a (Fig. 4a, 4b, 4c and Fig. 4d). The specific melting points were 85.98ºC for the E gene, 90.34ºC for the N gene and 83.0ºC for the Orf1ab gene.

Limit of detection

After ensuring an amplification for all the genes, we assessed the limit of detection of the test using synthetic RNA controls. The limit of detection proceeded as suggested by the FDA, namely, 10-fold serial dilutions of synthetic RNA material were used (Fig. 5a), and 20 replicates of the hypothetical limit (19 out of 20 replicates detected, Fig. 5b). This brings our limit of detection to the 3–4 copy/µl range since all copy number above 3 produced 20 in 20 positive results (data not shown).

Clinical validation

Having defined our detection limit using the synthetic control, the next step was to translate these results using clinical samples to have a more meaningful result about the performance of this testing method. Clinical samples were kindly provided by “Laboratório Nacional de Referência para o Vírus da Gripe e outros Vírus Respiratórios (LNRVG)” from “Instituto Nacional de Saúde Ricardo Jorge (INSA)”, Portugal. A total of 75 samples were tested where 25 were positive and 50 were negative, despite this our test manage to identify incorrectly defined samples as shown in Table 1. All positive samples were characterized with cycle threshold values using RT-qPCR (Table 2).

Table 1 COVsense testing method compared to the gold-standard technique.

		RT-qPCR result		Total
		Positive	Negative	
KIT RT-LAMP COVSENSE	Positive	26 (TP)	0 (FP)	26
	Negative	2 (FN)	47 (TN)	49
Total		28 (TP+FN)	47 (TN+FP)	75

Table 2 - Positive clinical samples tested and the results obtained by RT-PCR and our testing method. Cycle thresholds are represented under the specific amplified region ORF/N/E.COVsense testing method compared to the gold-standard technique.

Identification of sample	Sample type	Result RT-qPCR Fosun SARS CoV2	ORF	N	E	RT-LAMP-CRISPR/Cas12a
133999	Nasopharyngeal swab	Positive	11	10,03	12,6	Positive
134000		Positive	11,88	11	12	Positive
134402		Positive	10,46	9,04	11,14	Positive
134114		Positive	12,15	10,55	12,53	Positive
134118		Positive	25,73	25,9	26,24	Positive
134426		Positive	23,05	22,45	25,01	Positive
134482		Positive	27,22	25,44	26,47	Positive
134504		Positive	28,19	27,07	27,49	Positive
134519		Positive	23,55	22,31	22,73	Positive
134522		Positive	12,62	11,35	12,25	Positive
134579		Positive	15,19	14,33	16,16	Positive
134584		Positive	17	15,08	17,08	Positive
134627		Positive	13,09	11	14	Positive
134609		Positive	32,41	32,06	34,06	Positive
134776		Positive	30,14	24,9		Positive
134916		Positive	14,36	16,19	15,14	Positive
134917		Positive	24,51	24,4	25,14	Positive
134918		Positive	37,79	29,32	32	**Negative**
134919		Positive	13,15	12,53	14,06	Positive
134920		Positive	18,34	17,84	15,19	Positive
134921		Positive	20,61	19,62	21,12	Positive
134922		Positive	20,85	16,5	19,16	Positive
134924		Positive	26,11	24,51	26,05	Positive
134925		Positive		31,1	35,36	**Negative**
134926		Positive	25,26	24,03	25,37	Positive

Samples ranging from Ct 9 to a Ct of 37 were tested with excellent overall agreement and specificity for Ct below 35 (Fig. 6). Despite this, the sensitivity of this test is almost at the threshold of the gold-standard considering that samples are considered negative for the virus with a threshold above 37. These results provide our testing method with 100% of specificity since there were no false positive results among the negative samples that were tested, and 93% of positive agreement with a total agreement of 97% (Table 3). Rnase P tests were not made due to low sample volume.

Table 3 Agreement between the RT-qPCR test and the COVsense RT-LAMP CRISPR/Cas test.

Specificity of COVsense compared to RT-qPCR		
Positive percent agreement (%)	TP/(TP+FN) *100%	93%
Negative percent agreement (%)	TN/(TN+FP) *100%	100%
Total agreement PA (%)	(TP+TN)/(TP+TN+FN+FP) *100%	97%
Theoretical agreement Pe	[(TP+FP) (TP+FN) +(FN+TN) (FP+TN)]/(TP+TN+FN+FP)2	0,54
Coefficient K	(PA-Pe)/(1-Pe)	0,94

Table 4

Positive samples that were previously categorized as negative with diverse cycle threshold values.

Identification of sample	Sample type	Other respiratory viruses	PCR Fosun SARS CoV 2	ORF	N	E	COVsense result
ADR14	Nasopharyngeal swab	RSV A	Positive	-	32,5	34,85	Positive
ADR31		Rhinovirus	Positive	27,54	24,8	25,64	Positive
ADR35		Negative	Positive	15,93	13,7	15,33	Positive

The samples were poorly processed (probably due to being positive to other respiratory viruses) and were mislabelled and detected by our system. The RT-qPCR characterization showed samples with high Cts meaning that the most likely cause for negative results in the high Ct samples were probably due to

degradation (Table 4). Further tests using internal controls (Rnase P) were made for samples that tested negative and the results were negative (data not shown).

Detection of synthetic controls in saliva samples

We proceeded to test our assay in saliva samples. Two different buffers were tested for the inactivation and removal on inhibitors from saliva. The two buffers tested have slightly different compositions, the first one derives from the SHINE protocol (Fig. 7a) and is composed of 100 mM TCEP and 1 mM EDTA, pH = 8 and the second one is composed of 2.5 mM TCEP, 1 mM EDTA and 0.1% Tween-20 (Fig. 7b). Each saliva sample was heat inactivated by incubating at 65ºC for 30 min. A 2x solution of each of the buffers was mixed in a 1:1 proportion with saliva samples. These buffers were heat treated for 5 min at 95ºC and 22.5 µL of each was used to check for the presence of nucleases by incubating with the RnaseAlert substrate nuclease detection system (IDT), this takes advantage of a fluorescent RNA substrate in each reaction that will produce a signal if there are any Rnases in solution. In the positive control (PC) well, Rnase A is added. These solutions were incubated for 1h at 37ºC in a 384-well plate. It was possible to observe that there was no need to use Rnase Inhibitor when using these experimental conditions.

Saliva samples that were previously spiked with known amounts of inactivated virus and/or total swab controls were amplified via RT-LAMP and 2 µL of this reaction was used as input for 20 µL of the RNP mixture. The end point signal for the different concentrations is shown in Fig. 7c. Of note that the NTC reactions remained negative after 24h.

It was possible to achieve a limit of detection below 300 cp/µl for sample 1 (which was blind tested) while for other samples the limit was of 30 cp/µl (10X LOD for the extracted genetic material). There is the possibility that contaminants were present in sample 1, namely, poor accumulation of gargle leading to sample degradation, drinking coffee or eating before the 30 min asked to provide the sample.

In order to reduce contamination risks of the reaction vessel, workstation and equipment, there as the need to have a one-pot reaction. This hypothesis was put to the test, after optimization (data not shown) the best ratio was found to be 50 µL of Cas12a solution to 20 µL of RT-LAMP reaction. After 30 min of reaction time at 63ºC, the Eppendorf strip was briefly centrifuged (spin down) to mix the enzymatic solution located in the lid of the Eppendorf with the RT-LAMP reaction. This mixture was incubated at 37ºC for 20 min (Fig. 8).

The end point signal was found to have high significance after 20 min (Fig. 8a) and after 24h (Fig. 8b). These results provided the indication that it was possible to achieve the same detection limit as the 2-step method with high significance and no non-specific signal even after 24h.

Using the dedicated equipment, it is possible to process samples and determine the result needing only a centrifuge to mix the RNP reaction with the RT-LAMP amplification. Thus, in a short period of time we were able to create a proof-of-concept RT-LAMP/Cas12a SARS-CoV-2 molecular test that will be integrated inside a fully automated device. This test surpasses the current reagent and consumable shortage that was caused by the actual pandemic while still retaining a comparable limit of detection to

the gold-standard RT-qPCR. The only external equipment required to perform this kind of testing is a heat source (from 37ºC to 65ºC) a light source at 485nm and a spectrophotometer or a light sensor to detect the fluorescence signal. This testing method shows great potential to integrate the next generation of molecular diagnostics overtaking PCR as the gold standard to enable highly sensitive point-of-care technologies.

Conclusions

While RT-PCR has high turnaround times and requires experienced or specialized personnel to be performed, expensive equipment, and suitable facilities, isothermal amplification methods provide for point-of-care testing while retaining higher sensitivity than antigen tests in a low-resource environment. This innovative testing method poses several advantages when compared to other tests that are already commercialized, this is a one-pot testing method, there is no need to open any type of container minimizing the probability of having contaminations in the workspace; completely erases the need to use a thermocycler, highly versatile; and proved to be very sensitive (3 copies/µL) with a total assay time of 40 min.

Commercially available RT-LAMP kits provide reasonable detection of SARS-CoV-2 but without the needed robustness or specificity. We successfully implemented different reaction additives that had been previously described onto our assay (L-Proline and Guanidine HCL) allowing for a more consistent and robust response. By combining multiplex RT-LAMP amplification of 3 different genes of the SARS-CoV-2 virus with the simultaneous detection of these genes by CRISPR/Cas12a it was possible to achieve very high sensitivity while retaining 100% specificity thus eliminating the major problem of isothermal amplification methods which is non-specific amplification. The detection of synthetic RNA controls in saliva samples poses a great step forward in a less intrusive testing method that can be used more recurrently. This can have major implications on new diagnostic technologies since it is possible to reduce time of amplification without sacrificing specificity and bring testing capabilities to areas where they were not accessible before. The universal character of this testing method proves to have endless possibilities when it comes to detecting viral infection, bacteria, or other types of pathogens since the adaptation process of this method only requires changing primers and guide RNAs for different targets.

Materials And Methods

Nucleic acid preparation. SARS-CoV-2 target sequences were designed using the available genomic sequence from NCBI as of 30 January 2021. These sequences were then aligned with bat SARS-like CoV and MERS to verify cross-reactivity (Table A1). LAMP primers were based on previously published sequences. Considering currently used target sites by the CDC and WHO, gRNAs were designed for the E gene, S gene, and the ORF1ab as well as a sample control gRNA that that was designed for previously published RNase P POP7 RT-LAMP primers. These were either based on open-source sequences (gRNA for E and N and RNase P) that had already been published or designed by root (Orf1ab gRNA). For the in vitro testing a synthetic ssRNA control was used. Oligonucleotide DNA sequences were synthesized by

IDT with a yield of 25 nmole using standard desalting and then diluted to 100 µM of final concentration in nuclease-free water (IDT). The reporter molecule was an oligonucleotide functionalized with FAM (5´-end) and with BHQ (3'-end) using HPLC purification with a yield of 1µmole based on the sequence used by DETECTR. gRNAs were ordered from IDT with a yield of 2nmol and standard desalting all with 5' and 3' Alt-R end-blocking modifications and standard desalting with a stock solution of 50 µM.

5X Primer mixture with guanidine. LAMP primer mixtures (1X final, 10X stock diluted with guanidine to 5X) were assembled using a final concentration of 0.2 µM of F3/B3, 0.4µM of LF/LB, and 1.6µM of FIP/BIP in nuclease-free water. The stock solution of each primer (N/E/ORF1ab) 10X was mixed in equimolar proportions with guanidine (200mM in 5X primers mix and 40mM final concentration) to a concentration of 5X and this was the solution that was used for the simultaneous detection of the 3 different genes (Table A2).

RT-LAMP fluorescent assays. For the detection of SARS-CoV-2 RNA, several precautions were used to prevent sample contamination, such as using nuclease decontamination solution or RNase Away, filtered tips, and nuclease-free certified reagents in a dedicated clean bench as well as a laminar flow chamber with 20 min of UV decontamination when opening the plates was needed. For the RT-LAMP Vienna biocentre 12,13 reactions 11µL of tube 2A- LAMP MIX was mixed with 2.5 µL of tube 3A-SARS-CoV-2 As1 primer and with 2.5 µL of tube 4A-HNB dye and where referred, 0.4 µL of tube 7A- FLUO DYE (SYTO-9). Of this mixture, 16 µL were dispensed per well and 4µL of extracted RNA was added, vortexed, spined down, and incubated for 35 min at 63ºC. For the RT-LAMP mixture by SARS-CoV-2 Rapid Colorimetric LAMP Detection Assay Protocol (NEB #E2019) 12.5 µL of WarmStart Colorimetric LAMP 2X Master Mix with UDG, 2.5 µL of SARS-CoV-2 LAMP Primer Mix (N/E), 2.5 µL Guanidine Hydrochloride (40 mM of final concentration), 7.5 µL of nuclease-free-water and 2.0 µL of either sample nucleic acid (Extracted RNA), SARS-CoV-2 Positive Control (N gene) to a total reaction volume of 25 µL. When using WarmStart® LAMP Kit (DNA & RNA), for a final volume of 20.4 µL the reaction was assembled with 10 µL of WarmStart® Colorimetric LAMP 2X Master Mix (NEB #M1804), 4 µL of an equimolar 5X Primer mix (N/E/Orf1ab) with 200mM of Guanidine HCL, 0.7 mM of dUTP, 0.7mM of $MgSO_4$, 0.02 U/µL of UDG, 0.32 U/µL of Bst 2.0 WarmStart® DNA Polymerase (#M0538M), 0.4 µL of 50X LAMP dye and 0.92 µL of nuclease-free-water, to this mixture 4 µL of extracted RNA samples was added in different concentrations. These reactions were incubated at either 65ºC or 63ºC where specified for 30 min. For the real-time fluorescent detection of the LAMP result, a 384-well plate was used in QuantStudio™ 5 Real-Time PCR Instrument using 30 cycles of 1 min at the specified temperature using the standard FAM emission and excitation filters. Melt curve analysis was performed from 35ºC to 95ºC with a heating ramp of 0.5 ºC/s.

CRISPR assay. For the CRISPR-based detection system, we used Lba Cas12a from New England Biolabs (EnGen® Lba Cas12a (Cpf1) #M0653T). Firstly, Cas12-gRNA complexes were generated by pre-incubating LbaCas12a (50nM, final concentration) with the gRNA for each gene (50 nM final concentration for all – 1/3 proportion) for 20 min at 37ºC in 1X NEBuffer 2.1 for a final volume of 20 µL. After the incubation, the fluorescent reporter molecule was added at a final concentration of 4 µM and placed on ice. After the desired target amplification by RT-LAMP, 2 µL of each reaction is added to 20 µL

of the Cas12-gRNA mixture and incubated at 37ºC for 20 min. The real-time fluorescence acquisition was done using the Varioskan™ LUX multimode microplate reader using 20 cycles of 1 min.

In silico analysis. All SARS-CoV-2 sequences were aligned using Clustal omega using available genomes from GenBank (NCBI) as of January 2021. The specific target sites by LbaCas12a were compared with human coronavirus genomes (NC_045512.2), bat SARS-like CoV (NC_014470.1), and with MERS (NC_019843.3) to assess cross-reactivity. The LAMP primers were analysed via PrimerExplorer v.5 (https://primerexplorer.jp/e/) or based on previously published primers. All designed gRNAs were 100% specific (**Figure A1**, **Figure A2**, **Figure A3**) with Expect (E) values of $5x10^{-6}$ in the plus/plus strand, the E gene gRNA enables the detection of SARS-CoV-2 (100%) bat coronavirus (95.24%) and MERS (93.75%). All RT-LAMP primers have been previously validated as highly specific with minimal cross reactivity as confirmed by the in-silico analysis.

Human clinical sample collection and preparation. Clinical nasopharyngeal swab samples were kindly provided by Instituto Ricardo Jorge (LNRVG) either from patients infected with SARS-CoV-2 (or other respiratory viruses) or from RT-qPCR negative individuals. Sample RNA was extracted according to the manufacturer's protocol. All experiments were performed in accordance with relevant guidelines and regulations to work with human samples. Protocols were approved by Faculdade de Farmácia da Universidade de Lisboa and ethical committee CEISH 03/2021.

Real-time RT-qPCR assay. The determination of positive or negative samples was confirmed by RT-PCR using “Novel Coronavirus (2019-nCoV) RT-qPCR Detection Kit (Fosun 2019-nCoV qPCR), this kit sets the maximum cycle threshold at 36 for a positive signal. This assay was performed on the Biorad Real-Time PCR system instrument.

Analytical validation (LoD). LoD is the lowest detectable concentration at which around 95% of all true positive replicates test positive. The FDA recommends testing a dilution series of three replicates per concentration with inactivated controls and then confirm the final concentration with 20 replicates. The analytical validation of the detection kit was performed by using synthetic RNA controls provided by Twist Biosciences. From this stock, ten-fold serial dilutions were made until the detection limit was reached (2/3 true positives were detected). This limit was then tested in 20 replicates achieving a total of 19/20 for the initial sample solution of 3 copies/µL.

Clinical validation. The analysis was carried out on 75 anonymized nasopharyngeal swabs with negative (n = 50) and positive (n = 25) results by RT-qPCR provided by INSA. The samples were freshly extracted on the 23rd of August 2021 and remained stored in the laboratory until they were delivered to our team on the 24th of August 2021. Once received at -20°C, the 75 samples were analysed for the E, Orf1ab, N viral genes as well as internal control such as Rnase P, to determine sensitivity and specificity values, as well as repeatability and limit of detection (analytical sensitivity). All experimental protocols were approved by Instituto Ricardo Jorge, Laboratório Nacional de Referência para o Vírus da Gripe (LNRVG). All methods were carried out in accordance with relevant guidelines and regulations (**Figure A4**).

Processing of saliva samples. Saliva samples were retrieved from healthy, covid negative donors and kept at -20ºC. Each saliva sample was heat inactivated by incubating at 65ºC for 30 min. Two different buffers were tested, the first one is composed of 100 mM TCEP and 1 mM EDTA, pH = 8 and the second one is composed of 2.5 mM TCEP, 1 mM EDTA and 0.1% Tween-20. A 2x solution of each of the buffers was mixed in a 1:1 proportion with saliva samples that were previously spiked with known amounts of inactivated virus and/or total swab controls. These were heat treated for 5 min at 95ºC and 22.5 µL of each was used to check for the presence of nucleases by incubating with the RnaseAlert substrate nuclease detection system (IDT), this takes advantage of a fluorescent RNA substrate in each re-action that will produce a signal if there are any Rnases in solution. In the positive control (PC) well, Rnase A is added. These solutions were incubated for 1h at 37ºC in a 384-well plate. For the detection tests, these samples were spiked with SARS-CoV-2 RNA controls from Twist Biosciences and treated using the developed protocol (1:1 in 2X Buffer with a final concentration of 100mM TCEP and 1mM EDTA pH = 8 for 5 min at 95ºC). The processed samples were then spiked with different initial concentrations. Informed consent was obtained from all subjects and/or their legal guardian(s).

Statistical analysis. Data were analysed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ and “ns” is non-significant. The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Author contributions statement

D.F. and A.C. and J.G. conceived the experiment(s), D.F. conducted the experiment(s), D.F. analysed the results. All authors reviewed the manuscript. JG funding for this work was supported by SAICTCOVID/72538/2020 - 02/SAICT/2020-SAICT (Projetosde IC&T Testar com Ciência). iMed was supported by UIDP/04138/2020 and UIDB/04138/2020 (Fundacao para a Ciencia e Tecnologia).

Additional information

The authors declare no competing interests.

Availability of Data and Materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

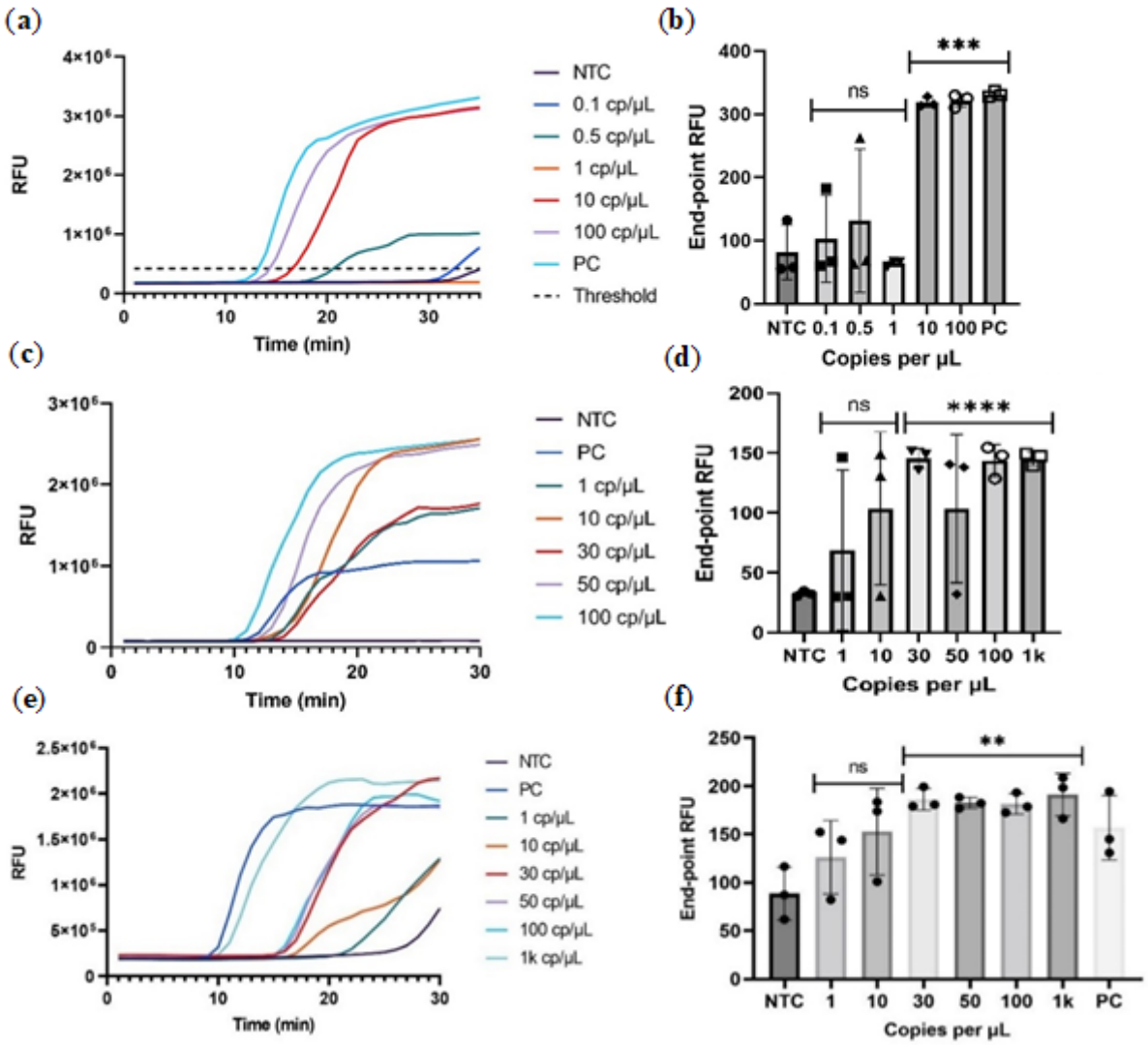

Figure 1

Real-time fluorescence for the NEB RT-LAMP reactions using the N/E primer set (a), the As1 primer set (c) and the N/E/As1 combined primer set (e). End point fluorescence measurements for the respective primer sets (b), (d) and (f). Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant

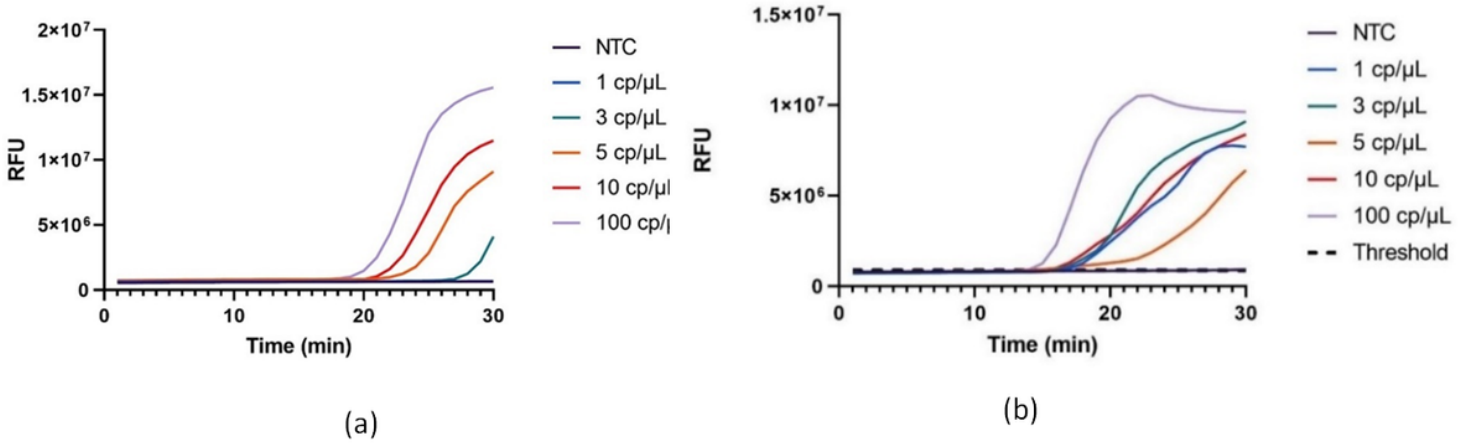

Figure 2

(a) Real-time fluorescence signal for the amplification of synthetic RNA controls using a 3-primer set with 40 mM of Guanidine HCL in the RT-LAMP reaction. (b) Amplification of synthetic RNA controls using a 3-primer set with 40 mM of Guanidine HCL and extra Bst 2.0 polymerase.

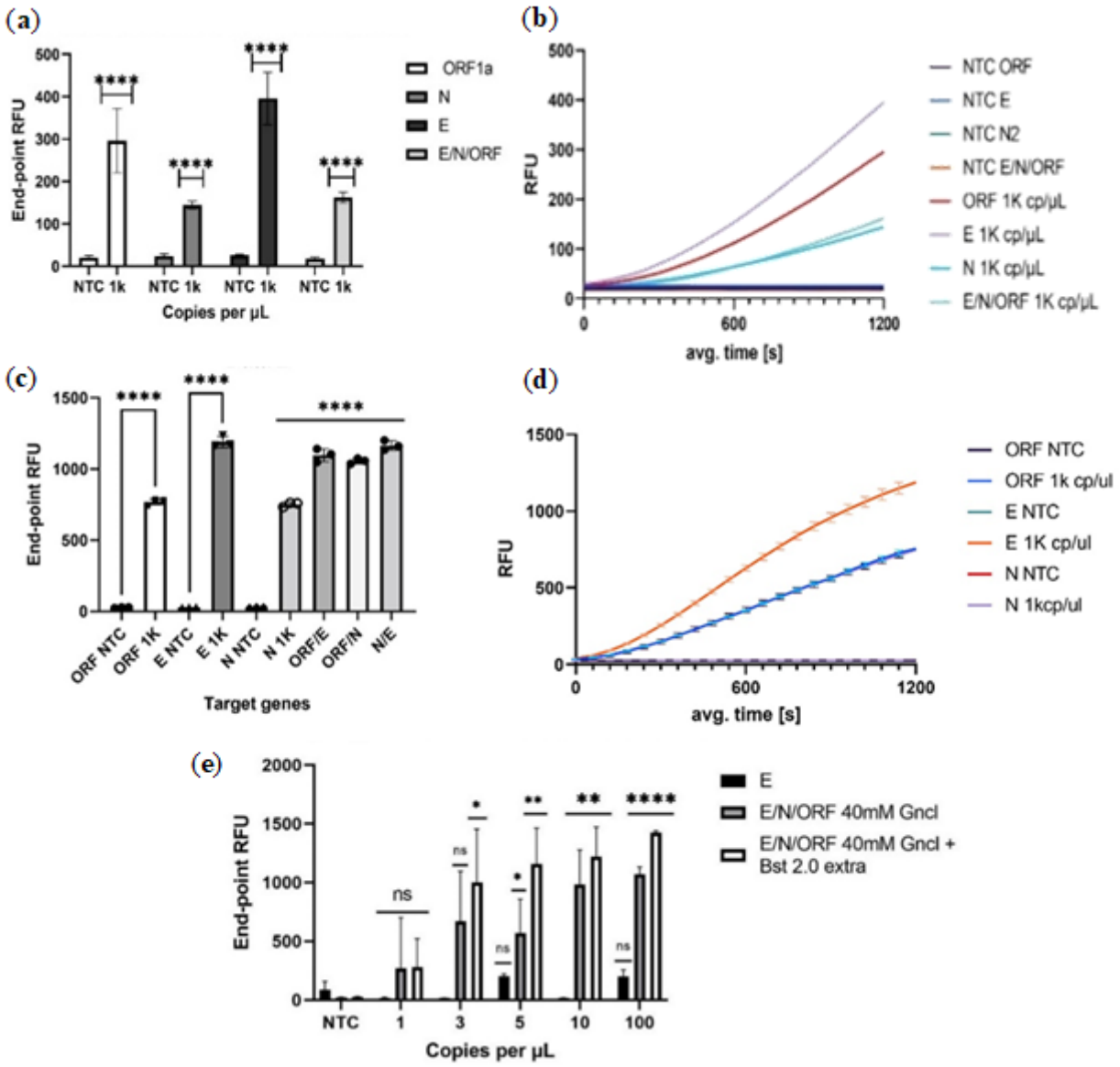

Figure 3

Detection of Sars-CoV-2 in the Cas12a reaction. (a). End point fluorescence of the non-optimized reaction and the respective kinetics (b). (c) Detection of individual genes amplified by RT-LAMP by Cas12a using the optimized version and respective kinetic profile (d). (e) Limit of detection using a 3-primer set with Guanidine hydrochloride and with extra Bst 2.0 polymerase. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant.

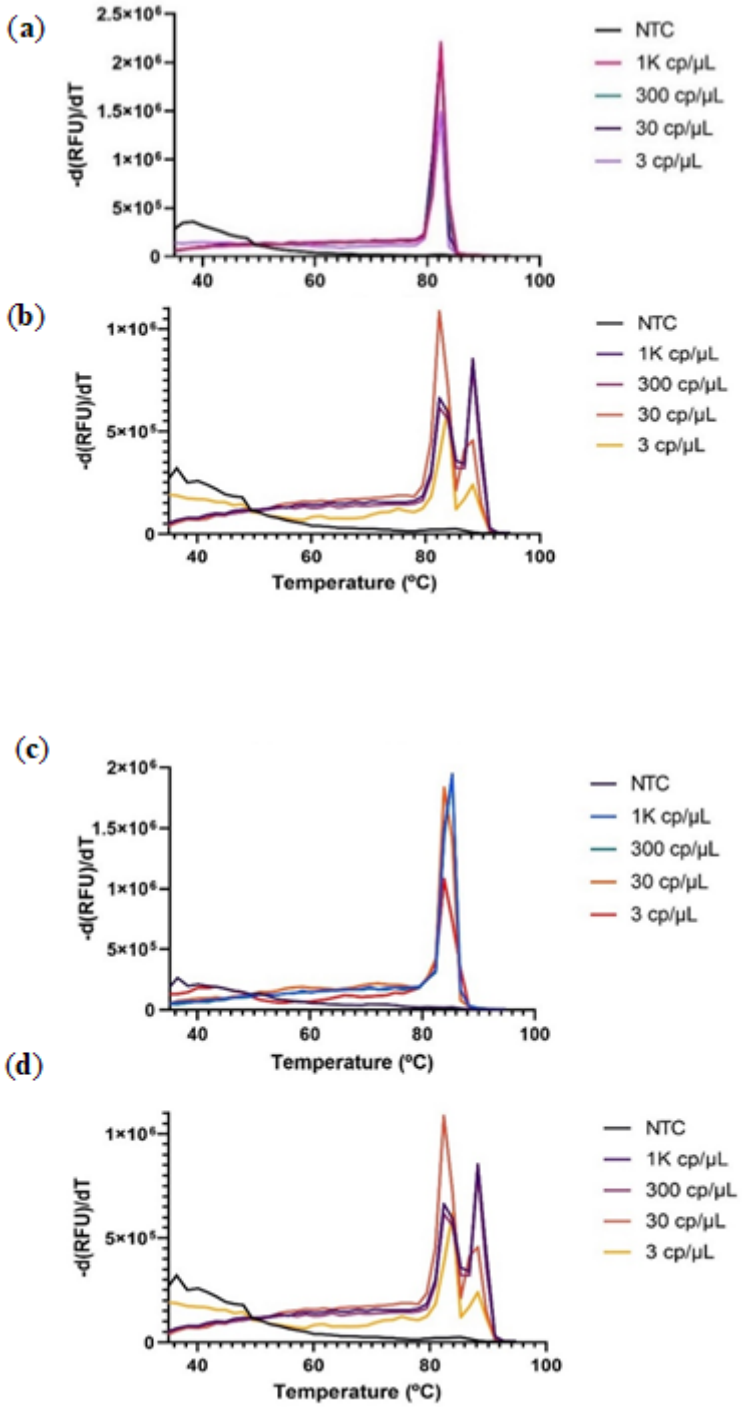

Figure 4

Individual genes were amplified via RT-LAMP using different amounts of synthetic RNA resulting in the corresponding melting curve obtained with N primers (a), E primers (b) and As1 primers (c). Multiplex amplification (d) via RT-LAMP using different amounts of synthetic RNA resulting in the corresponding melting curve to validate the successful amplification using an intercalating dye from 35ºC to 95 ºC with a heating ramp of 0.5ºC/s.

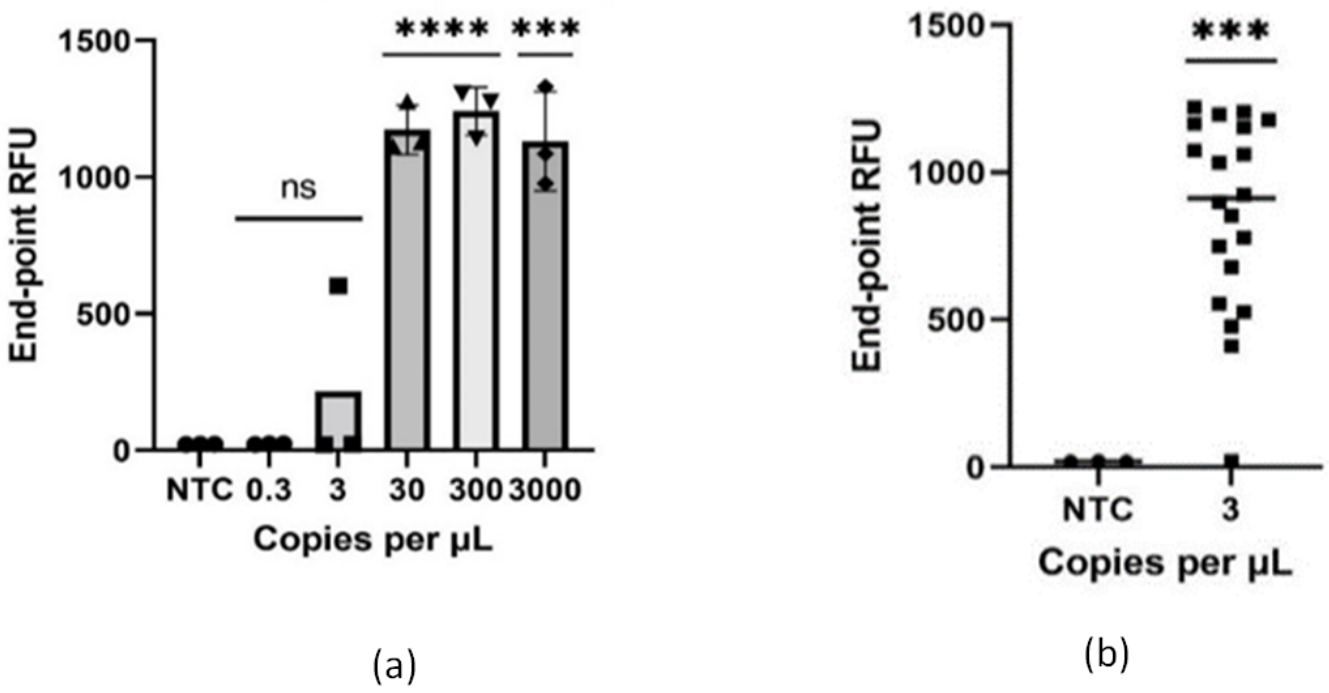

Figure 5

Limit of detection as recommended by the FDA. (a). End point detection of the different synthetic viral loads. (b) 20 replicates at this concentration revealed the detection of 19/20 indicating that this is the number of copies that is detected 95% of the time and thus our limit of detection. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant.

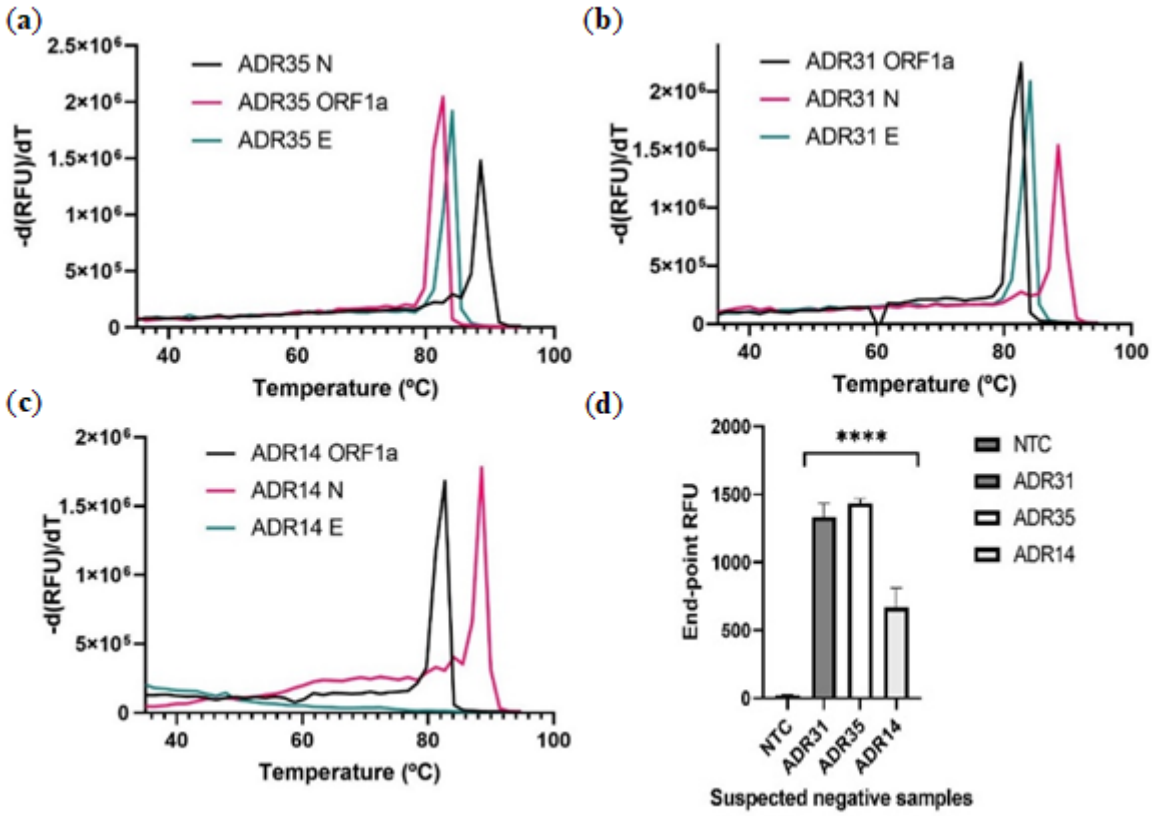

Figure 6

Samples that were considered SARS-CoV-2 positive by INSA were further analysed and thus did not correspond to false positive since there was always amplification in 2 genes. Melting curve for sample ADR35 (a), ADR31 (b) and ADR14 (c) using the primers sets for the 3 different genes B. End-point fluorescent signal measured in the Cas12a reaction. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant.

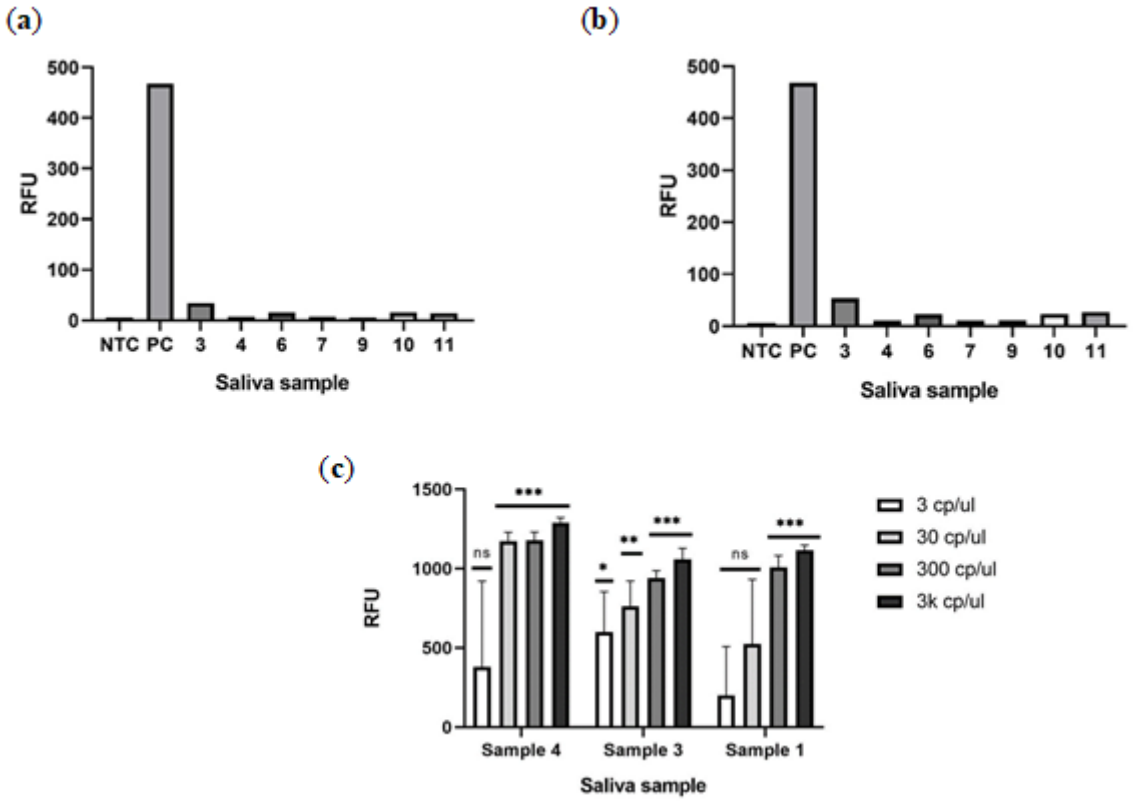

Figure 7

RnaseAlert substrate nuclease detection system reaction for the different saliva samples using the SHINE buffer (a) and the Tween-20 buffer (b) in a 1:1 proportion. (c) End-point fluorescence signal in the Cas12a-mixture of spiked saliva samples processed using the SHINE buffer. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant.

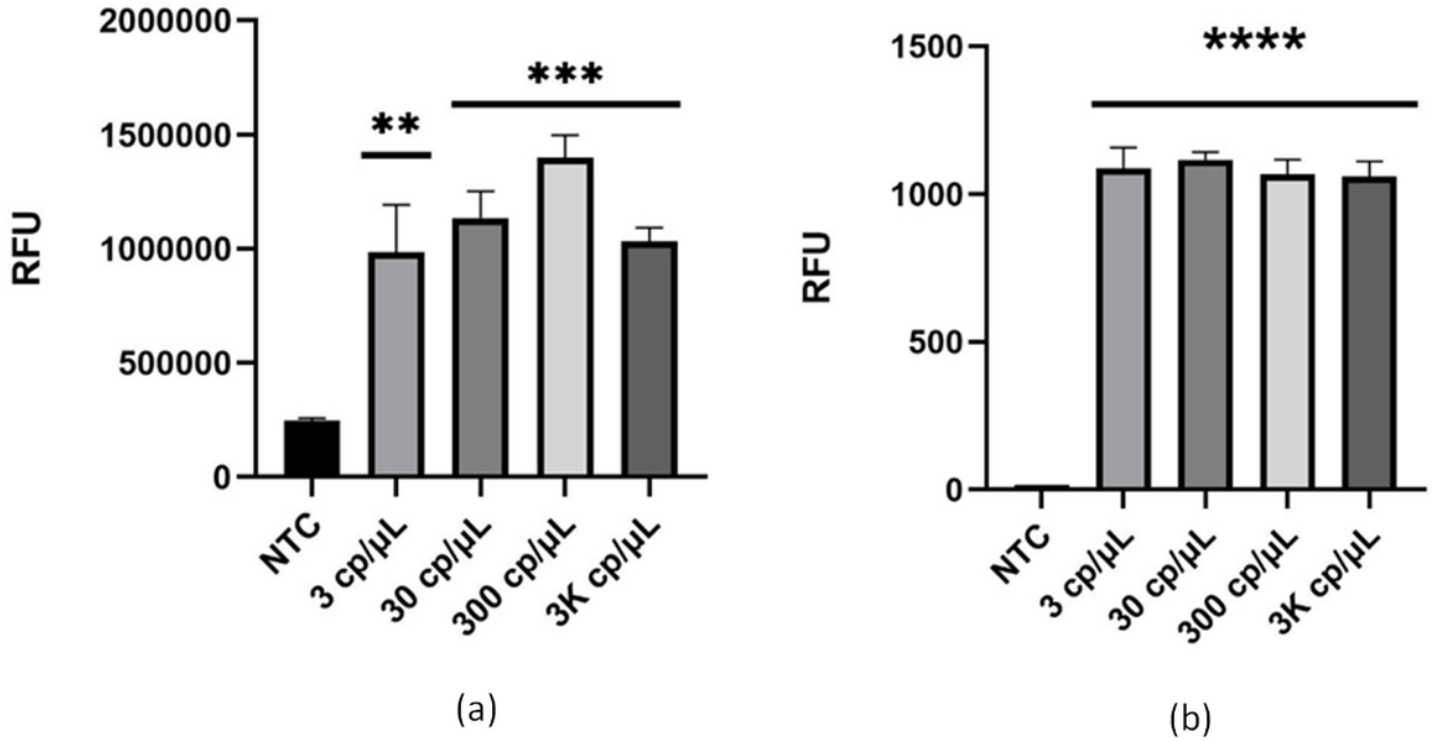

Figure 8

End point RFU signal using a dedicated equipment (a) after 20 min and Varioskan Lux multimode plate reader and (b) after 24h of RNP reaction time at room temperature. Statistical significance was determined by unpaired two-tailed t-test and all data were shown as mean ± S.D of 3 replicates. Asterisks indicate ** P<0.01; *** P<0.001; **** P<0.0001 and “ns” is non-significant.

Supplementary Files

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