

# A molecular test based on RT-LAMP for rapid, sensitive and inexpensive colorimetric detection of SARS-CoV-2 in clinical samples

**Catarina Amaral**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Wilson Antunes**

Centro de Investigação da Academia Militar, Unidade Militar Laboratorial de Defesa Biológica e Química

**Elin Moe**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Américo G. Duarte**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Luís M. P. Lima**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Cristiana Santos**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Inês L. Gomes**

Centro de Investigação da Academia Militar, Unidade Militar Laboratorial de Defesa Biológica e Química

**Gonçalo S. Afonso**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Ricardo Vieira**

Centro de Investigação da Academia Militar, Unidade Militar Laboratorial de Defesa Biológica e Química

**Helena Sofia S. Teles**

Centro de Medicina Naval - Marinha Portuguesa

**Marisa Reis**

Centro de Medicina Naval - Marinha Portuguesa

**Manuel A. Ramalho Silva**

Hospital das Forças Armadas

**Ana Margarida Henriques**

Instituto Nacional de Investigação Agrária e Veterinária, I.P., Laboratório de Virologia

**Miguel Fevereiro**

Instituto Nacional de Investigação Agrária e Veterinária, I.P., Laboratório de Virologia

**M. Rita Ventura**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

**Mónica Serrano**

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa  
**Catarina Pimentel** (✉ [pimentel@itqb.unl.pt](mailto:pimentel@itqb.unl.pt))

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

---

## Research Article

**Keywords:** SARS-CoV-2, RT-LAMP, colorimetric detection

**Posted Date:** May 4th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-180877/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at Scientific Reports on August 12th, 2021.  
See the published version at <https://doi.org/10.1038/s41598-021-95799-6>.

1 **A molecular test based on RT-LAMP for rapid, sensitive and inexpensive**  
2 **colorimetric detection of SARS-CoV-2 in clinical samples**

3  
4

5 Catarina Amaral<sup>1</sup>, Wilson Antunes<sup>2</sup>, Elin Moe<sup>1</sup>, Américo G. Duarte<sup>1</sup>, Luís M. P. Lima<sup>1</sup>,  
6 Cristiana Santos<sup>1</sup>, Inês L. Gomes<sup>2</sup>, Gonçalo S. Afonso<sup>1</sup>, Ricardo Vieira<sup>2</sup>, Helena Sofia  
7 S. Teles<sup>3</sup>, Marisa S. Reis<sup>3</sup>, Manuel A. Ramalho da Silva<sup>4</sup>, Ana Margarida Henriques<sup>5</sup>,  
8 Miguel Fevereiro<sup>5</sup>, M. Rita Ventura<sup>1</sup>, Mónica Serrano<sup>1\*</sup>, Catarina Pimentel<sup>1\*</sup>

9  
10 <sup>1</sup> Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa,  
11 Av. República, 2780-157 Oeiras, Portugal.

12 <sup>2</sup> Centro de Investigação da Academia Militar (CINAMIL), Unidade Militar Laboratorial de  
13 Defesa Biológica e Química (UMLDBQ), Av. Dr. Alfredo Bensaúde, 1849-012 Lisboa,  
14 Portugal.

15 <sup>3</sup> Centro de Medicina Naval - Marinha Portuguesa, Alfeite, 2810-001 Almada, Portugal.

16 <sup>4</sup> Hospital das Forças Armadas, Azinhaga dos Ulmeiros, 1649-020 Lisboa, Portugal.

17 <sup>5</sup> Instituto Nacional de Investigação Agrária e Veterinária, I.P., Laboratório de Virologia, Av.  
18 República, Quinta do Marquês, 2780-157 Oeiras, Portugal.

19 \* email: serrano@itqb.unl.pt; pimentel@itqb.unl.pt

20  
21  
22  
23  
24  
25

1 **Abstract**

2

3 Until there is an effective implementation of COVID-19 vaccination program, a robust  
4 testing strategy, along with prevention measures, will continue to be the most viable  
5 way to control disease spread. Such a strategy should rely on disparate diagnostic  
6 tests to prevent a slowdown in testing due to lack of materials and reagents imposed  
7 by supply chain problems, which happened at the beginning of the pandemic.

8 In this study, we have established a single-tube test based on RT-LAMP that enables  
9 the visual detection of less than 100 viral genome copies of SARS-CoV-2 within 30  
10 minutes. We benchmarked the assay against the gold standard test for COVID-19  
11 diagnosis, qRT-PCR, using 177 nasopharyngeal RNA samples. For  $Ct \leq 32$ , the RT-  
12 LAMP assay had a sensitivity of 100% and a specificity of 96.1%. Additionally, we set  
13 up a RNA extraction-free RT-LAMP test capable of detecting SARS-CoV-2 directly  
14 from saliva samples, albeit with lower sensitivity. The saliva was self-collected and  
15 the collection tube remained closed until inactivation, thereby ensuring the protection  
16 of the testing personnel. As expected, RNA extraction from saliva samples increased  
17 the sensitivity of the test. To lower the costs associated with RNA extraction, we  
18 performed this step using an alternative protocol that uses plasmid DNA extraction  
19 columns. We also produced the enzymes needed for the assay and established an  
20 in-house-made RT-LAMP test independent of specific distribution channels. Finally,  
21 we developed a new colorimetric method that allowed the detection of LAMP  
22 products by the visualization of an evident color shift, regardless of the reaction pH.

23

24

## 1 **Introduction**

2

3 A robust population-scale testing strategy for SARS-CoV-2 based on rapid, reliable,  
4 decentralized and affordable diagnostic tests is of utmost priority to guide public  
5 health interventions. This testing approach aligned with measures such as mask  
6 wearing, frequent hand washing and social distancing may be enough to prevent and  
7 contain major outbreaks while COVID-19 vaccination programs are in progress.

8 The gold standard of COVID-19 testing is quantitative RT-PCR (qRT-PCR), which  
9 detects the genetic material of SARS-CoV-2 in nasopharyngeal (NP) samples.  
10 Although very reliable, qRT-PCR diagnostics are complex, laborious and expensive,  
11 and its worldwide use caused, in the early stages of the pandemic, a shortage of  
12 reagents needed for sample collection and viral RNA extraction. Thus, qRT-PCR-  
13 based tests, in their current format, are unlikely to serve the purposes of a mass  
14 testing of the population.

15 Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that  
16 allows rapid and sensitive detection of a specific gene <sup>1-3</sup>. LAMP merged with reverse  
17 transcription (RT-LAMP) has been successfully used for the detection of several  
18 respiratory RNA viruses <sup>4-8</sup>, including SARS-CoV-2 (reviewed in <sup>9</sup>). RT-LAMP is a  
19 powerful alternative to qRT-PCR due to its high specificity and sensitivity, cost-  
20 effectiveness, and fast turnaround time (typically 30 minutes). In RT-LAMP, the  
21 amplification of the genetic material of the virus occurs at a constant temperature  
22 and, therefore, diagnostic tests based on RT-LAMP can be carried out anywhere with  
23 basic resources, as they only require a heat block or a water bath set to a single  
24 temperature. The reaction products can be analyzed by means of conventional DNA-  
25 intercalating dyes, agarose gel electrophoresis, UV-light illumination, or real-time  
26 fluorescence <sup>10</sup>. Alternatively, end-point colorimetric readouts are also possible  
27 through the detection of reaction by-products, such as pyrophosphate and protons,  
28 which are released during DNA polymerization, after the incorporation of  
29 deoxynucleotide triphosphates. LAMP colorimetric methods detect the turbidity,  
30 triggered by the accumulation of magnesium pyrophosphate <sup>1</sup>, or color changes,  
31 occurring when complexometric indicators <sup>3,11</sup>, pH sensitive dyes <sup>12</sup> or even DNA-  
32 intercalating dyes <sup>13-15</sup> are incorporated into the reaction. The simple technical and  
33 instrumental requirements of colorimetric RT-LAMP tests make them extremely  
34 attractive for point-of-care (POC) use and implementation in low-resource settings.

1 Colorimetric RT-LAMP has been successfully used for detection of SARS-CoV-2 in  
2 NP fluids from COVID-19 patients <sup>15-24</sup>.

3 Recently, it was shown that SARS-CoV-2 could be detected in the saliva of infected  
4 individuals, highlighting salivary tests as valuable alternatives for COVID-19  
5 diagnosis <sup>25-27</sup>. Saliva-based testing has numerous advantages over NP sampling,  
6 especially in a mass screening scenario. It can be performed easily and non-  
7 invasively, thus minimizing patient discomfort, and it does not require specialized  
8 personnel or the use of protective equipment, which saves time and reduces costs.  
9 For these reasons, saliva qRT-PCR and RT-LAMP tests for SARS-CoV-2 detection  
10 have been widely explored in recent months <sup>28-31</sup>.

11

12 In the current study, we have established and evaluated a RT-LAMP colorimetric test  
13 for SARS-CoV-2 detection from RNA samples extracted from the NP fluid, or directly  
14 from the saliva, of COVID-19 patients. We have also developed a new colorimetric  
15 detection method based on a complexometric indicator that, when merged to LAMP,  
16 is capable of detecting SARS-CoV2 with great analytical sensitivity. In addition, we  
17 have produced the enzymes needed for the test and implemented an in-house-made  
18 assay fully independent of commercial reagents.

19 With this work, we join efforts with many other authors who, in the last months, have  
20 been testing and validating alternative tests for the detection of SARS-CoV-2 in order  
21 to make the molecular diagnosis of COVID-19 more accessible and to facilitate its  
22 large-scale implementation, even in settings that lack economic or infrastructural  
23 resources.

24  
25

## 1 **Results**

2

### 3 **Sensitivity of two different RT-LAMP colorimetric setups**

4 The main components of the RT-LAMP colorimetric reaction are two enzymes (a  
5 reverse transcriptase (RT) and a strand displacement polymerase), a colorimetric  
6 dye (phenol red) and a primer set (typically composed of six primers)<sup>12</sup>. To detect  
7 SARS-CoV-2 using RT-LAMP, we took advantage of the primer set previously  
8 validated *in vitro* by Zhang *et al.*<sup>24</sup> and tested, on clinical specimens from large  
9 cohorts of COVID-19 patients, by several other authors<sup>16-19</sup>. The primer set (N-A)  
10 targeted the N gene, which encodes the nucleocapsid protein and has the most  
11 abundant expression of subgenomic mRNA during infection<sup>32-35</sup>.

12 We tested two different assay formats. In one format, we used the WarmStart  
13 Colorimetric LAMP 2× Master Mix (New England Biolabs), which includes all the  
14 reagent components with the exception of the primers. In the other, we purchased  
15 the separate enzymes (RTx and *Bst* 2.0) from New England Biolabs, while the  
16 reaction buffer with the colorimetric dye (phenol red) were prepared in-house as  
17 described by Tanner *et al.*<sup>12</sup>. The analytical sensitivities of these two setups were  
18 evaluated and compared by assaying in parallel tenfold serial dilutions of an *in vitro*  
19 transcribed N-gene RNA standard (IVT RNA), starting from 10<sup>5</sup> copies down to 10  
20 copies (per 20 µL reaction), at tenfold intervals (Fig. 1A). Color changes from pink  
21 (negative) to yellow (positive) were registered after a 30-minute incubation period at  
22 65°C, as we found that for extended periods (up to 60 minutes), negative controls  
23 often turned yellowish. The amplification of the IVT RNA was confirmed by agarose  
24 gel electrophoresis (Fig. 1B). Ten replicates were analyzed per assay format (Fig.  
25 1C) and IVT RNA dilutions were simultaneously analyzed by qRT-PCR (Fig. 1D). The  
26 limit of detection (LoD) was reliably found to be between 100–1000 viral copies for  
27 the assay using the WarmStart Colorimetric LAMP 2× Master Mix (Fig. 1A), whereas  
28 for that using the separate components the LoD was consistently one Log10 lower  
29 (10–100 copies). However, for half of the replicates, a tenfold lower LoD was  
30 achieved for both test formats (Fig. 1C). Such stochastic detection efficiency has  
31 been reported by others (1), and therefore we defined 100–1000 (WarmStart  
32 Colorimetric LAMP 2× Master Mix) and 10–100 (reaction with separate components)  
33 as the robust limits of detection.

34 For the same serial dilution range, the qRT-PCR assay was able to consistently  
35 detect down to 10 copies per reaction (mean Ct=35.22) (Fig. 1D). Compared to qRT-

1 PCR, the RT-LAMP assay, depending on the test setup, detected up to ten- or one  
2 hundred-fold less copies of viral RNA. As the RT-LAMP format using the separate  
3 components was consistently more sensitive, we decided to choose this setup in  
4 subsequent assays.

5

### 6 **Sensitivity and specificity of the colorimetric RT-LAMP assay in detecting viral** 7 **RNA from the nasopharyngeal fluid**

8 We investigated whether the RT-LAMP assay, using separate components, could be  
9 used to accurately detect SARS-CoV-2 in clinical samples. For that purpose, we  
10 tested a set of surplus RNA samples extracted from the nasopharyngeal (NP) fluid of  
11 177 individuals who were previously tested for COVID-19, using the standard clinical  
12 qRT-PCR testing. The samples comprised 126 RNA samples that tested positive  
13 (qRT-PCR positive,  $Ct \leq 40$ ) and 51 samples that tested negative (qRT-PCR negative,  
14  $Ct \geq 40$ ). As shown in Fig. 2A, after incubation for 30 minutes at 65°C, a pink to  
15 yellow color change was visualized in all RT-LAMP reactions estimated to have more  
16 than 100 RNA molecules present in the reaction (qRT-PCR positive,  $Ct \leq 32$ , Fig. 1C),  
17 which is in agreement with the observed experimental sensitivity (Fig. 1A). We found  
18 two false positives, i.e. two qRT-PCR negative samples that scored positive in the  
19 RT-LAMP assay (Table 1). Thus, the overall specificity of the assay was 96.1% (CI:  
20 87-99%) and the sensitivity for samples with  $Ct \leq 32$  was 100% (CI: 94.7-100%). For  
21 lower viral load, as measured by qRT-PCR ( $Ct > 32$ ), the assay showed a decrease in  
22 diagnostic sensitivity (Table 1, Fig. 2B).

23 Overall, these results indicate a robust performance of the colorimetric RT-LAMP  
24 assay across a broad range of purified RNA samples.

25

### 26 **Sensitivity of the colorimetric RT-LAMP assay in detecting SARS-CoV-2 in** 27 **saliva samples**

28 We next optimized our RT-LAMP assay for direct detection of SARS-CoV-2 in saliva  
29 samples. To reduce the risk associated with handling samples containing infectious  
30 viral particles, saliva was self-collected into a tube and placed at 95°C for 30 minutes,  
31 for inactivation. This simple heat inactivation procedure has been shown to enable an  
32 effective genetic detection of SARS-CoV-2 by other authors<sup>30,31</sup>. After a brief  
33 centrifugation step that significantly improved assay reliability (data not shown), the  
34 supernatant was diluted with TE, to buffer basal pH differences in saliva, and



1 immediately analyzed or stored at -80°C. Lalli *et al.* have shown that TE is LAMP-  
2 compatible and does not affect the assay sensitivity<sup>29</sup>.

3 We determined the LoD of the assay using both the IVT RNA standard and viral  
4 SARS-CoV-2 particles spiked into healthy human saliva to simulate clinical samples.  
5 We were able to detect 100 IVT RNA copies (Fig. 3A) and 24 SARS-CoV-2 viral  
6 particles (Fig. 3B) per reaction in only 30 minutes after inactivation, using our RT-  
7 LAMP protocol. Since at this sensitivity the assay would detect the typical viral load of  
8 SARS-CoV-2 found in the saliva of COVID-19 patients (100-1000 genomes per µl)<sup>36</sup>,  
9 we proceeded to test the clinical samples.

10 Saliva and matched NP swab specimens of 49 individuals infected with SARS-CoV-2  
11 (as previously determined by qRT-PCR) were collected and analyzed by RT-LAMP  
12 (saliva) and qRT-PCR (NP fluid). In addition, 15 saliva samples of healthy donors  
13 were tested by RT-LAMP. Saliva samples were self-collected as described above,  
14 and individuals were asked not to eat or drink before testing. A set of 10 of the 49  
15 COVID-19-positive patients was asked to induce salivation by placing the tongue on  
16 the salivary sublingual glands. For this group, we could only detect SARS-CoV-2  
17 sequences in the saliva of one patient using the direct RT-LAMP assay (Fig. 4A).  
18 However, after RNA extraction, 8 out of 10 individuals were identified as being SARS-  
19 CoV-2-positive. We assumed that by stimulating salivation we were diluting the saliva  
20 viral load, which might have accounted for a high number of false negatives.  
21 Corroborating this idea, for all other positive samples where salivation was not  
22 induced, we obtained a good correlation with the qRT-PCR results (Fig. 4B and C),  
23 as 33 out of 39 samples were identified as positive samples, with no false positives  
24 registered. Therefore, the direct RT-LAMP assay had a sensitivity of 85% (CI: 70–  
25 93%) for saliva samples with matched NP swabs with Ct ≤28 (Fig. 4C). Reaction  
26 volumes, but not saliva amounts, were scaled up to increase the assay sensitivity  
27 (Fig.4B).

28 All saliva samples that were falsely negative by direct RT-LAMP were positive after  
29 RNA extraction (Fig. 4B). This step increases by 4–9 times the estimated cost of the  
30 assay (1€). Inspired by the work of Yaffe *et al.*<sup>37</sup>, to keep RT-LAMP affordable, we  
31 tested whether we could use silica columns routinely used in molecular biology  
32 laboratories to purify bacterial plasmids (mipreps), to extract viral RNA from saliva  
33 samples. As shown in Fig. 4D, false negative samples were found to be positive after  
34 RNA purification using this method, with an estimated cost per RT-LAMP test of 2€.

35

## 1 **Development of an in-house-made colorimetric RT-LAMP**

2 Aiming to establish a colorimetric RT-LAMP test fully independent of commercial  
3 suppliers, we produced the two enzymes needed for the assay and benchmarked  
4 them against commercial alternatives using IVT RNA of SARS-CoV-2.

5 As for the strand displacement polymerase, the gene encoding the large (Klenow)  
6 fragment of *Geobacillus stearothermophilus* was synthesized, with codon optimized  
7 for expression in *E. coli*, and inserted into the pET28+ vector. After a simple 2-step  
8 purification protocol, we ended up with 250 µL of *Bst* LF, at a concentration of 7.6  
9 mg/mL. We next determined the LoD of the assay combining 1 µL of the purified *Bst*  
10 LF, 50-fold diluted (0.15 µg per 20 µL reaction), the in-house-made colorimetric  
11 reaction buffer, and RTx (New England Biolabs). This semi-commercial assay  
12 consistently detected 1–10 copies of the SARS-CoV-2 N gene per reaction (Fig. 5A).  
13 We found that, under our colorimetric conditions, *Bst* LF outperformed *Bst* 2.0 (New  
14 England Biolabs) (Fig 5A and 1A). The amount of the produced *Bst* LF was enough  
15 to perform 12 500 tests at that analytical sensitivity (1-10 copies).

16 Alternatives to the commercial RTx were also explored. We started by testing several  
17 non-thermostable reverse transcriptases (from NZYtech and Roche), but it was not  
18 possible to detect LAMP products with an acceptable sensitivity (less than 10<sup>6</sup> viral  
19 IVT RNA copies per reaction, data not shown). We also expressed and purified the  
20 MashUP RT enzyme (clone available at <https://pipettejockey.com>) that, when  
21 combined with *Bst* 2.0, was able to detect down to 10 IVT viral RNA copies (Fig. 5B),  
22 a LoD similar to the one obtained with the commercial enzyme (Fig. 5A). The  
23 MashUP purification consists of a single-step protocol, and sufficient enzyme was  
24 obtained to perform 500 assays (0.5 µL corresponding to 3.4 µg/µL were used  
25 directly in the reaction).

26 Finally, we combined the produced enzymes (*Bst* LF and MashUP) with the  
27 homemade colorimetric reaction mixture and assessed (i) the LoD of the assay (Fig.  
28 5C) and (ii) whether this setup could identify SARS-CoV-2 N-gene sequences in the  
29 RNA extracted from the NP fluid of COVID-19 patients (Fig. 5D). Our in-house-made  
30 assay successfully detected SARS-CoV-2 viral sequences in all the three COVID-19  
31 patients' samples (Fig. 5D). Moreover, when using patients' saliva, processed as  
32 described above, instead of NP RNA, the assay was also capable of identifying  
33 SARS-CoV-2 infected patients (Fig. 5E). Corroborating the work of Alekseenko *et al.*  
34 <sup>38</sup>, these results clearly indicate that, using simple expression and purification  
35 protocols and home-made buffers, it is possible to establish a colorimetric assay, fully

1 independent of specific supply chains, that efficiently detects SARS-CoV-2 RNA  
2 sequences from clinical specimens.

3

4 **A new colorimetric method for detection of RT-LAMP amplification products**

5 The strong and evident color shift observed with phenol red renders this pH-sensitive  
6 dye much preferred for end-point colorimetric detection of LAMP products. However,  
7 when the phenol red method is used with crude samples, interference of the sample  
8 pH with the assay readout is often observed. Indeed, when establishing the direct  
9 RT-LAMP saliva protocol, we had to discard one sample due to the initial acidification  
10 of the reaction, as a strong color shift to yellow was observed immediately after  
11 sample addition into the reaction mixture. Although several colorimetric indicators are  
12 available for detection of LAMP products <sup>3,11-15</sup>, the pale color shift they produce,  
13 which is difficult to distinguish by the naked eye, has certainly restrained their wide  
14 use. To overcome these limitations, we developed a new colorimetric detection  
15 method based on the complexometric indicator, murexide (MX), which forms a  
16 complex with divalent zinc ( $Zn^{2+}$ ) <sup>39</sup>. In the absence of  $Zn^{2+}$ , MX has a pink color,  
17 whereas in the presence of the divalent cation it turns yellow. Because  
18 pyrophosphate (PPi) forms a strong complex with zinc, we reasoned that the PPi  
19 released during DNA polymerization would displace  $Zn^{2+}$  cations from MX, inducing a  
20 color change from yellow to pink. By mimicking the reaction components in a test  
21 tube containing the Zn-MX complex, an evident color shift from yellow to pink was  
22 observed immediately after PPi addition (Fig. 6A). Unfortunately, we found that Zn,  
23 but not MX, strongly inhibited the LAMP reaction (data not shown), making it  
24 impossible to use Zn-MX in a one-step colorimetric assay. Therefore, after an  
25 incubation period at 65°C for 30 minutes, the tubes were opened and MX (0.5 mM)  
26 and  $ZnCl_2$  (2.5 mM) were added to the reaction. To avoid carryover problems due to  
27 the post-amplification opening of the tubes, this step was performed in a separate  
28 room.

29 Using the in-house produced enzymes, we first compared the sensitivity of Zn-MX  
30 with that of phenol red using IVT RNA (Fig. 6B and C). Like phenol red, Zn-MX  
31 showed an evident color difference depending on the presence (pink) or absence  
32 (yellow) of LAMP amplification. Moreover, the method enabled the clear detection of  
33 SARS-CoV-2 in crude saliva samples of nine COVID-19 positive patients (Fig. 6D),  
34 whereas with phenol red the viral genetic material was only identified in eight of these  
35 samples (Fig. 6E).

1 **Discussion**

2 Widespread testing, preferably based on different supply chains, is required to curtail  
3 the ongoing pandemic. To address that need, we have in this work evaluated a  
4 LAMP-based colorimetric test to rapidly detect SARS-CoV-2 in RNAs extracted from  
5 patient's NP fluids, using a single tube protocol. The assay also allows for detection  
6 of the virus directly from patient's saliva with minimal processing and increased  
7 protection of the testing personnel. We also showed that using simple expression  
8 and purification protocols together with homemade buffers, it is possible to establish  
9 an inexpensive colorimetric assay, fully independent of specific supply chains, that  
10 efficiently detects SARS-CoV-2 RNA.

11 While not as sensitive as the reference diagnostic method for COVID-19, qRT-PCR,  
12 the simplicity, turnaround time and low associated costs of our test make it an  
13 attractive and efficient tool for infection control. According to existing literature, the  
14 LoD of the test is sufficient to identify individuals with viral titers high enough to  
15 transmit the virus (300-1000 viral copies per  $\mu\text{L}$ ) <sup>27,40,41</sup>. This test sensitivity is  
16 understood to be adequate for surveillance and screening of the asymptomatic  
17 population. The availability of such a testing solution is therefore of great importance,  
18 as infectiousness peaks occur before or at the symptoms onset <sup>42</sup>. Indeed, the rapid  
19 evolution of COVID-19 has been partly attributed to transmissions occurring through  
20 people who are presymptomatic or asymptomatic <sup>43</sup>; efforts to implement a strategy  
21 enabling communities to test asymptomatic individuals require urgent attention and  
22 testing tools to support it.

23 Several authors have recently shown that the use of different primer sets boosts RT-  
24 LAMP sensitivity, possibly due to better primer efficiency and/or higher target  
25 abundance. Also different saliva treatment protocols, combining certain chemicals  
26 and proteinase K, have been shown to improve SARS-CoV-2 detection in saliva  
27 samples <sup>16,21,22,28,29,31</sup>. Thus, we reason that there is still room to improve the  
28 sensitivity of our test.

29 As expected, RNA extraction greatly improved the saliva test sensitivity, by  
30 increasing the concentration of the viral sequences in the sample. Many other  
31 authors have reported similar findings <sup>21,22,44,45</sup> and extensive efforts have been made  
32 to establish alternative protocols that enable RNA enrichment using fast and  
33 inexpensive methodologies <sup>21,22</sup>. Here we showed that RNA extraction using common  
34 plasmid DNA extraction columns is an economical way to concentrate and purify viral  
35 RNA from saliva samples.

1 To eliminate the impact of acidic saliva samples on the test readout, we have  
2 developed a new colorimetric reading, independent of changes in the pH of the  
3 LAMP reaction. The method uses a divalent zinc salt (such as  $ZnCl_2$ ) and the  
4 complexometric indicator murexide to form a transient complex (Zn-MX). The  
5 presence of PPI, a by-product of the reaction, is indicated by the indicator  
6 displacement method, since  $Zn^{2+}$  forms a more stable complex with PPI and thus  
7 releases murexide. As the presence of zinc inhibits the amplification reaction, the  
8 metal can only be added at the end of the reaction, thus requiring the tubes to be  
9 opened post-amplification. This procedure poses the threat of carryover  
10 contaminations, very common in LAMP reactions<sup>46,47</sup>, which leads to false positives.  
11 We therefore do not anticipate that the Zn-MX method, in its current formulation, can  
12 be used routinely in a molecular diagnostic laboratory. However, the molecular  
13 saliva-based tests currently available for COVID-19, whose workflow already  
14 demands opening the LAMP reaction tube, may certainly benefit from our method<sup>48</sup>.  
15 Additionally, the method can be safely used in closed systems using microfluidic  
16 diagnostic cartridges, similar to the one recently described by Ganguli *et al.*<sup>49</sup>.  
17 Overall, this study, while addressing some of the testing bottlenecks imposed by the  
18 current pandemic, reinforces RT-LAMP as a powerful method for sensitive and  
19 inexpensive molecular diagnosis of COVID-19 that can be easily deployable in limited  
20 resource settings.  
21  
22

1 **Materials and Methods**

2

3 **Sample Collection, Processing and storage**

4 Clinical specimens were collected at Hospital das Forças Armadas and processed in  
5 Laboratório de Bromatologia e Defesa Biológica (Unidade Militar Laboratorial de  
6 Defesa Biológica e Química). Saliva specimens (~1ml) were self-collected into sterile  
7 tubes (50 mL or 1.5 mL). Patients were asked not to eat or drink before testing. NP  
8 swab-matched samples were collected in parallel and placed in 3 ml Universal Viral  
9 Transport Media. Tubes containing clinical specimens were decontaminated with an  
10 alcohol-based solution and identified. After collection, samples were kept at 4°C for  
11 2-4 days or processed immediately. Samples were inactivated by incubation at 95°C  
12 for 5 minutes (NP swabs) or 30 minutes (saliva samples). Salivas were centrifuged at  
13 5000 g for 5 minutes and 200 µL of the supernatant were diluted in TE 10x (1x, final  
14 concentration) and frozen at - 80°C until analysis. The saliva pellets were also frozen.

15

16 **RNA extraction from clinical samples**

17 Total viral RNA was extracted from 140 µl of NP deactivated samples using Viral  
18 RNA Mini Kit (QIAGEN) and eluted in 60 µl of RNase free water, to ensure the RNA  
19 elution buffer has no impact of pH in RT-LAMP reactions. As for saliva samples, total  
20 RNA (from the pellets) was isolated using the RNeasy Mini Kit (QIAGEN) following  
21 the manufacturer's instructions or the LogSpin method <sup>37</sup> as described by the  
22 authors. Briefly, the pellet was mixed by vortexing with 250 µL a guanidine-based  
23 solution (8M guanidine-HCl, 20 mM MES hydrate and 20 mM EDTA). The mixture  
24 was centrifuged at 16000 g for 5 min and the supernatant was mixed with 250 µL of  
25 100% ethanol, and loaded into the ZR plasmid miniprep columns (ZYMO Research).  
26 The column was washed twice with 450 µl of 3 M Na-Acetate and 320 µl of 70%  
27 ethanol. RNA was eluted in 30 µl of water.

28

29 **SARS-CoV-2 RNA standard**

30 To prepare the SARS-CoV-2 RNA standard, the N gene was amplified from the  
31 plasmid 2019-nCoV\_N\_Positive Control (Integrated DNA Technologies) with a T7-  
32 promoter-containing primer (5' –  
33 TAATACGACTCACTATAGGatgtctgataatggaccccaaaa – 3') and the reverse primer  
34 (5'- ttaggcctgagttgagtcagc-3'), then the product was *in vitro* transcribed using the  
35 HiScribe T7 High Yield RNA Synthesis Kit, NEB), according to the manufacturer's

1 instructions. Template DNA was removed using Turbo DNase (Invitrogen) and RNA  
2 was then purified using the RNeasy Mini Kit (QIAGEN). Standard RNA copy numbers  
3 were calculated from concentration measured using Take3 from Epoch from Biotek  
4 and confirmed using a Ultrospec2100pro (Amersham Biosciences).

#### 6 **Virus isolation and spike experiments**

7 SARS-Cov-2 isolate, BetaCoV/Portugal/ICV1006/2020, was obtained at INIAV from a  
8 patient confirmed positive for SARS-CoV-2 by qRT-PCR. Virus isolation and  
9 production of the virus stock were accomplished in Vero E6 cells (African green  
10 monkey kidney cells, catalog no.ATCC CRL-1586) maintained in Eagle's minimum  
11 essential medium (MEM) supplemented with 10 % fetal bovine serum (FBS),  
12 penicillin (100 U/ml) and streptomycin (100 mg/ml), at 37°C in a 5% carbon dioxide  
13 atmosphere. The infectivity titer of the viral stock prepared from infected cell culture  
14 supernatants was determined by a standard plaque assay. Aliquots of saliva (500 µl)  
15 were spiked with decreasing numbers of plaque forming units (pfus) of isolate  
16 ICV1006 and used to evaluate the limit of detection of the saliva RT-LAMP assay.

#### 18 **qRT-PCR**

19 SARS-CoV-2 N-gene and an internal control (RNase P) were amplified by qRT-PCR  
20 using the TaqMan 2019-nCoV Assay Kit v1 (Termofisher) with TaqMan Fast Virus 1-  
21 step Master Mix (Termofisher) and the CFX96 thermocycler (BioRad), according to the  
22 manufacturer's instructions.

#### 24 **RT-LAMP assays**

25 RT-LAMP reaction was performed in a total volume of 20 µL containing the following  
26 components: 8 U *Bst* 2.0 (NEB), 7.5 U RTx (NEB) and 1x colorimetric buffer mix (1.6  
27 µM FIP/BIP primers, 0.4 µM LF/LB primers, 0.2 µM F3/B3 primers Gene N-A<sup>24</sup>, 10  
28 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Merck), 50 mM KCl (BDH), 8 mM MgSO<sub>4</sub> (BDH), 0.1% Tween 20, 0.2  
29 mM Phenol Red (Sigma), 1.4 mM each dNTP (NZYTech)). For the in-house-made  
30 assay, we used the same colorimetric buffer mix, 0.5 µL of MashUP RT (6.8 mg/ml)  
31 and 1 µL of *Bst* LF (7.6 mg/ml) 50x diluted. WarmStart colorimetric LAMP 2x master  
32 mix (M1800S, NEB) was also used with the above final primer concentration.

33 When the complexometric indicator MX-Zn was used, samples were assembled as  
34 described above, but without phenol red. After 30 min, 2 µL of 5 mM Murexide and 1  
35 µL of 50 mM of ZnCl<sub>2</sub> were added to the reaction, in a post-LAMP workspace. All

1 reactions were performed in a thermocycler at 65°C and pictures were taken at the  
2 indicated time points. Figures depicting the readout of the RT-LAMP assays are  
3 representative of three independent experiments.

4

#### 5 **Zinc-Murexide colorimetric method**

6 All reagents obtained from commercial sources in analytical grade. Analytical  
7 solutions were prepared in ultrapure grade water from a Milli-Q system, as follows:  
8 MOPS buffer pH=7.4 at 20 mM, magnesium chloride (MgCl<sub>2</sub>) at 47.5 mM, zinc  
9 chloride (ZnCl<sub>2</sub>) at 47.1 mM, sodium pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) at 50 mM, ATP at 25  
10 mM, and murexide (MX) at 0.5 mM. The MX solution was prepared immediately  
11 before use or otherwise kept frozen. Samples (1 mL), simulating the starting  
12 conditions of the RT-LAMP assay, contained 8 mM of magnesium chloride and 1.4  
13 mM of ATP, buffered at pH=7.4 with 10 mM of MOPS. To these samples were added  
14 a few drops of a MX solution to attain a suitable color intensity, which turned the  
15 samples violet, indicating that MX was in the free form. Addition of ZnCl<sub>2</sub> at 8 mM to  
16 the samples rendered them orange, indicating a change of the indicator to its  
17 complexed form. Finally, titration of pyrophosphate into the samples caused a color  
18 change back to pink from ca. 16 mM, pointing to a release of the indicator caused by  
19 the binding of zinc to pyrophosphate. These color changes demonstrated that MX is  
20 a suitable colorimetric indicator to detect pyrophosphate in presence of magnesium.

21

#### 22 **Expression and purification of Bst1 Klenow**

23 The gene encoding the klenow fragment of *Bst1* (UniProt sequence P52026, residue  
24 291 – 876) was synthesized (codon optimized for expression in *E.coli*) and inserted  
25 into the pET28+ vector with nucleotides encoding an N-terminal 6HisTag and a TEV  
26 cleavage site (Genescript). The resulting plasmid was used for transformation of *E.*  
27 *coli* BL21 (DE3) pLysS. Overnight pre-cultures (10 ml) were grown at 37°C and used  
28 to inoculate 1L Power Broth (Molecular Dimensions) with 100 µg mL<sup>-1</sup> ampicillin and  
29 50 µg mL<sup>-1</sup> kanamycin. The culture was grown at 37°C until OD<sub>600</sub> reached 0.7-0.9. At  
30 this point, the culture was moved to 18°C and expression was induced by adding 0.5  
31 mM isopropyl-β-d-thiogalactopyranoside (IPTG). After overnight expression, the cells  
32 were harvested by centrifugation at 7548 g for 30 minutes at 4°C, flash frozen and  
33 stored at -20°C. Upon protein purification, the cells were resuspended in 20 mL  
34 extraction buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mg/mL  
35 DNase I, 1 mg/mL lysozyme and one tablet EDTA free proteinase inhibitor (Roche))



1 and subjected to multiple freeze/thaw cycles (alternating room temperature water  
2 bath and liquid nitrogen). The lysate was cleared by centrifugation at 48385 g for 30  
3 min at 4°C and the supernatant was carefully removed and added to a 5 mL HisTrap  
4 HP purification column (Cytiva), previously equilibrated in buffer A (150 mM NaCl, 50  
5 mM Tris-HCl pH 7.5). The protein was eluted over a 10 CV gradient from 5 – 100%  
6 buffer B (buffer A with 0.5 M Imidazol). Fractions containing Bst1 Klenow were  
7 identified by SDSPAGE, pooled and dialyzed overnight in 2 L buffer A in the  
8 presence of TEV (1:20) at 4°C. The dialyzed and TEV cleaved protein was thereafter  
9 added to a 5 mL HisTrap column and eluted in the Flow Through (due to the removal  
10 of the HisTag). The HisTag free Bst1 Klenow was thereafter desalted through a  
11 HiTrap desalting column (Cytiva) followed by a final purification step on a 5 ml HiTrap  
12 Heparin HP column (Cytiva), to remove eventual residual DNA bound to the protein.  
13 The protein was eluted over a 10 CV gradient in buffer B2 (buffer A and 1 M NaCl).  
14 Fractions containing BstKlenow was identified by SDSPAGE, pooled, concentrated to  
15 7.6 mg/ml by Amicon Ultra-15 concentration filter units (10 kDa cut off, Millipore) and  
16 stored at -80°C.

17

### 18 **Expression and purification of MashUP reverse transcriptase**

19 The MashUp RT plasmid (kindly provided by <https://pipettejockey.com>), which  
20 encodes a modified Feline Leukemia Virus Reverse Transcriptase (RT) and plasmid  
21 pGTf2 that encodes for a chaperon were co-transformed into *E. coli* BL21 (DE3)  
22 competent cells and plated on L-Broth (LB) agar (NZYTech) plates containing 50  
23 µg/mL kanamycin and 30 µg/mL chlorophenicol. Overnight cultures were inoculated  
24 with fresh transformants and grown at 37 °C, 150 RPM in LB selective medium.  
25 Subsequently, the overnight culture was diluted 100x in Terrific Broth (TB). The cells  
26 were grown at 37 °C, 150-170 RPM until OD 600 nm reach 0.8-1.0. Then,  
27 temperature was lowered to 18 °C and protein expression induced with 0.5 mM IPTG  
28 and 5 ng/mL tetracycline, for the RT and chaperone, respectively, and grown  
29 additionally for 18 h at 18 °C. The cells were harvested by centrifugation at 4500 × g  
30 for 10 min at 4 °C and resuspended in MashUp-RT lysis buffer (25 mM Tris-HCl pH  
31 8, 300 mM NaCl, 10% glycerol, 40 mM imidazole, 0.5% Triton X-100), supplemented  
32 with one tablet of Complete EDTA-free protease inhibitor cocktail (one unit per 1 L).  
33 Cells were disrupted by French press and the extract was clarified by centrifugation  
34 at 100 000 xg, 90 minutes at 4°C. The supernatant was loaded into an IMAC column  
35 equilibrated with lysis buffer. The column was washed with the same buffer and the

1 adsorbed proteins were eluted from the column with 25 mM Tris-HCl pH 8, 300 mM  
2 NaCl, 10% glycerol, 500 mM imidazole, 0.5% Triton X-100. Protein was concentrated  
3 in an Ammicon ultrafiltration device with a 30 kDa cutoff. Total protein present in the  
4 sample was quantified by BCA assay (6.8 mg/mL) using albumin as a standard.

#### 6 **Ethics statement**

7 The Director of the Hospital das Forças Armadas (HFA) approved all experimental  
8 procedures, which were carried out following the guidelines of the HFA Ethics  
9 Committee. The study was conducted in accordance with the European Statements  
10 for Good Clinical Practice and the declaration of Helsinki of the World Health Medical  
11 Association. Informed consent was obtained from all participants.

#### 13 **Acknowledgments**

14 We thank the members of the COVID-19 task force of ITQB NOVA and Mariana  
15 Pinho for helpful discussions and suggestions. In particular, we thank Claudio M.  
16 Soares and Adriano O. Henriques for their unconditional support and invaluable help  
17 in making this work possible. The authors acknowledge the assistance of Paula  
18 Chicau, ITQB NOVA, in the development of the saliva test and are grateful to Chuck  
19 Farah, São Paulo University, for the pGTf2 plasmid. This work was supported by (i)  
20 Project LISBOA-01-0145-FEDER-007660 (“Microbiologia Molecular, Estrutural e  
21 Celular”) funded by FEDER funds through COMPETE2020 – “Programa Operacional  
22 Competitividade e Internacionalização” (POCI), (ii) the European Union’s Horizon  
23 2020 research and innovation programme under grant agreement No 810856 and (iii)  
24 “Fundação para a Ciência e a Tecnologia” (FCT) through programme IF  
25 (IF/00124/2015) to C.P and through the project “*DETECT: Development of an Easy,*  
26 *fast-Track and Economical Colorimetric Test for autonomous national diagnosis of*  
27 *COVID-19*” Ref 433\_613549914 (20/7/153), attributed to C.P., under the scope of the  
28 2<sup>nd</sup> edition of the programme RESEARCH4COVID19.

#### 31 **Author contributions**

32 Conceptualization: CA and CP. Performed the experiments: CA, WA, CS, ILG, GSA,  
33 RV, MS, CP. Enzyme expression and purification: EM and AGD. Colorimetric method  
34 development: CA, MRV, LMPL, CP. Virus isolation: AMH and MF. Sample collection:  
35 HSST, MSR, MARS. Writing – original draft: CP, Writing – review & editing: CA, MS,  
36 WA, EM, AGD, LMPL, ILG, MF, RV, CP. Project administration: MS and CP. Funding  
37 acquisition: CP.

#### 39 **Competing interests**

40 The authors declare no competing interests.

1 **Tables**

2

3

4 Table 1. RT-LAMP sensitivity across different ranges of Ct values

<b>RT-LAMP</b>			
<b>qRT-PCR</b> (Ct values range)	<b>True positives</b>	<b>False negatives</b>	<b>% Sensitivity (95 % CI)*</b>
[0 – 40]	84	42	67.2 (58.56 - 74.81)
[0 – 32]	68	0	100 (94.65 - 100)
[32 – 35]	17	20	45.95 (31.04 – 61.62)
[35 – 40]	11	27	28.95 (17 – 44.76)

5 \*Wilson's binominal confidence interval

6

7

8

9

10

## References

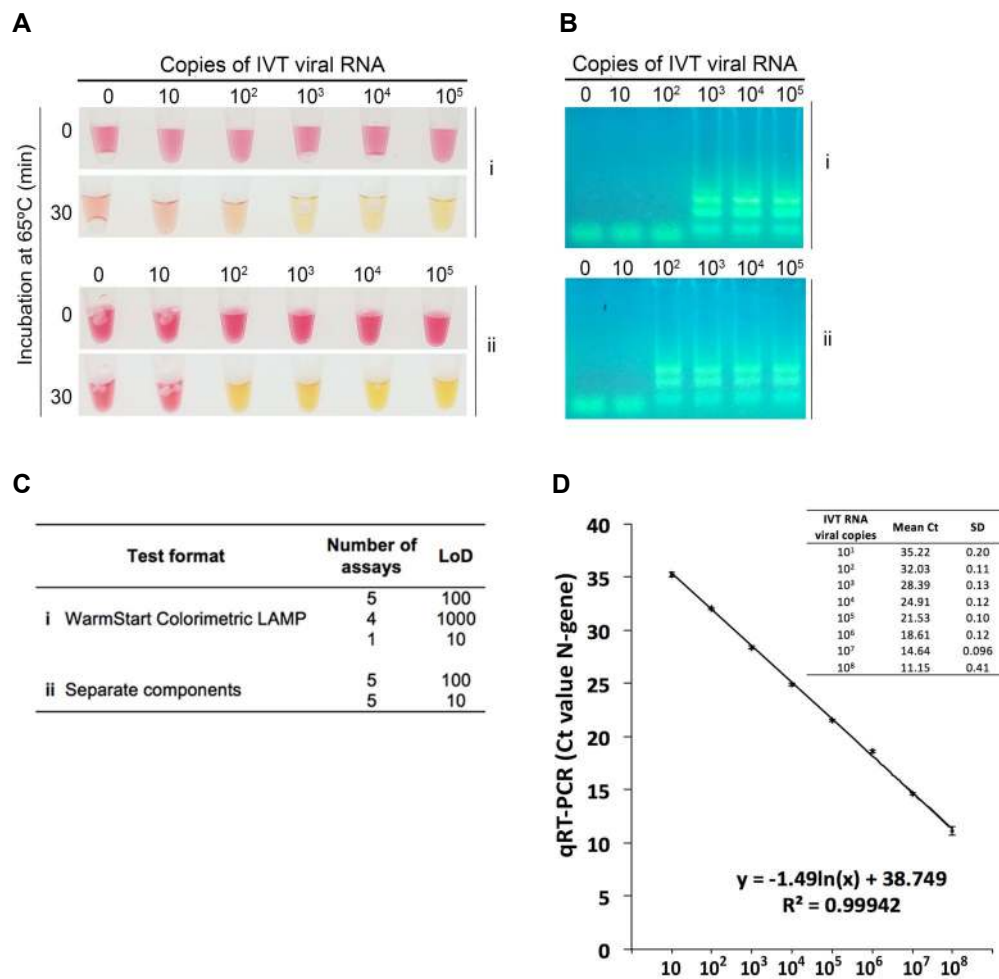
- 1 Nagamine, K., Hase, T. & Notomi, T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* **16**, 223-229, doi:10.1006/mcpr.2002.0415 (2002).
- 2 Notomi, T. *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**, E63, doi:10.1093/nar/28.12.e63 (2000).
- 3 Tomita, N., Mori, Y., Kanda, H. & Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* **3**, 877-882, doi:10.1038/nprot.2008.57 (2008).
- 4 Ahn, S. J. *et al.* Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *BMC Infect Dis* **19**, 676, doi:10.1186/s12879-019-4277-8 (2019).
- 5 Bhadra, S. *et al.* Real-time sequence-validated loop-mediated isothermal amplification assays for detection of Middle East respiratory syndrome coronavirus (MERS-CoV). *PLoS One* **10**, e0123126, doi:10.1371/journal.pone.0123126 (2015).
- 6 Hong, T. C. *et al.* Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *J Clin Microbiol* **42**, 1956-1961, doi:10.1128/jcm.42.5.1956-1961.2004 (2004).
- 7 Jayawardena, S. *et al.* Loop-mediated isothermal amplification for influenza A (H5N1) virus. *Emerg Infect Dis* **13**, 899-901, doi:10.3201/eid1306.061572 (2007).
- 8 Lee, S. H. *et al.* One-Pot Reverse Transcriptional Loop-Mediated Isothermal Amplification (RT-LAMP) for Detecting MERS-CoV. *Front Microbiol* **7**, 2166, doi:10.3389/fmicb.2016.02166 (2016).
- 9 Thompson, D. L., Y. Recent progress in RT-LAMP enabled COVID-19 detection. *Sensors and Actuators Reports* **2**, doi:10.1016/j.snr.2020.100017 (2020).
- 10 Quyen, T. L., Ngo, T. A., Bang, D. D., Madsen, M. & Wolff, A. Classification of Multiple DNA Dyes Based on Inhibition Effects on Real-Time Loop-Mediated Isothermal Amplification (LAMP): Prospect for Point of Care Setting. *Front Microbiol* **10**, 2234, doi:10.3389/fmicb.2019.02234 (2019).
- 11 Goto, M., Honda, E., Ogura, A., Nomoto, A. & Hanaki, K. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* **46**, 167-172, doi:10.2144/000113072 (2009).
- 12 Tanner, N. A., Zhang, Y. & Evans, T. C., Jr. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques* **58**, 59-68, doi:10.2144/000114253 (2015).
- 13 Fischbach, J., Xander, N. C., Frohme, M. & Glokler, J. F. Shining a light on LAMP assays--a comparison of LAMP visualization methods including the novel use of berberine. *Biotechniques* **58**, 189-194, doi:10.2144/000114275 (2015).
- 14 Lamb, L. E., Bartolone, S. N., Ward, E. & Chancellor, M. B. Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. *PLoS One* **15**, e0234682, doi:10.1371/journal.pone.0234682 (2020).
- 15 Park, G. S. *et al.* Development of Reverse Transcription Loop-Mediated Isothermal Amplification Assays Targeting Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). *J Mol Diagn* **22**, 729-735, doi:10.1016/j.jmoldx.2020.03.006 (2020).

- 1 16 Anahtar, M. N. *et al.* Clinical assessment and validation of a rapid and sensitive  
2 SARS-CoV-2 test using reverse-transcription loop-mediated isothermal  
3 amplification. *medRxiv*, 2020.2005.2012.20095638,  
4 doi:10.1101/2020.05.12.20095638 (2020).
- 5 17 Buck, M. D. *et al.* Standard operating procedures for SARS-CoV-2 detection by a  
6 clinical diagnostic RT-LAMP assay. *medRxiv*, 2020.2006.2029.20142430,  
7 doi:10.1101/2020.06.29.20142430 (2020).
- 8 18 Butler, D. J. *et al.* Shotgun Transcriptome and Isothermal Profiling of SARS-CoV-  
9 2 Infection Reveals Unique Host Responses, Viral Diversification, and Drug  
10 Interactions. *bioRxiv*, 2020.2004.2020.048066, doi:10.1101/2020.04.20.048066  
11 (2020).
- 12 19 Dao Thi, V. L. *et al.* A colorimetric RT-LAMP assay and LAMP-sequencing for  
13 detecting SARS-CoV-2 RNA in clinical samples. *Sci Transl Med* **12**,  
14 doi:10.1126/scitranslmed.abc7075 (2020).
- 15 20 Huang, W. E. *et al.* RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2.  
16 *Microb Biotechnol* **13**, 950-961, doi:10.1111/1751-7915.13586 (2020).
- 17 21 Kellner, M. J. *et al.* A rapid, highly sensitive and open-access SARS-CoV-2  
18 detection assay for laboratory and home testing. *bioRxiv*,  
19 2020.2006.2023.166397, doi:10.1101/2020.06.23.166397 (2020).
- 20 22 Rabe, B. A. & Cepko, C. SARS-CoV-2 detection using isothermal amplification  
21 and a rapid, inexpensive protocol for sample inactivation and purification. *Proc*  
22 *Natl Acad Sci U S A* **117**, 24450-24458, doi:10.1073/pnas.2011221117 (2020).
- 23 23 Yu, L. *et al.* Rapid Detection of COVID-19 Coronavirus Using a Reverse  
24 Transcriptional Loop-Mediated Isothermal Amplification (RT-LAMP) Diagnostic  
25 Platform. *Clin Chem* **66**, 975-977, doi:10.1093/clinchem/hvaa102 (2020).
- 26 24 Zhang, Y. *et al.* Rapid Molecular Detection of SARS-CoV-2 (COVID-19) Virus  
27 RNA Using Colorimetric LAMP. *medRxiv*, 2020.2002.2026.20028373,  
28 doi:10.1101/2020.02.26.20028373 (2020).
- 29 25 To, K. K. *et al.* Temporal profiles of viral load in posterior oropharyngeal saliva  
30 samples and serum antibody responses during infection by SARS-CoV-2: an  
31 observational cohort study. *Lancet Infect Dis* **20**, 565-574, doi:10.1016/S1473-  
32 3099(20)30196-1 (2020).
- 33 26 To, K. K. *et al.* Consistent Detection of 2019 Novel Coronavirus in Saliva. *Clin*  
34 *Infect Dis* **71**, 841-843, doi:10.1093/cid/ciaa149 (2020).
- 35 27 Wyllie, A. L. *et al.* Saliva or Nasopharyngeal Swab Specimens for Detection of  
36 SARS-CoV-2. *N Engl J Med* **383**, 1283-1286, doi:10.1056/NEJMc2016359  
37 (2020).
- 38 28 Dudley, D. M. *et al.* Optimizing direct RT-LAMP to detect transmissible SARS-  
39 CoV-2 from primary nasopharyngeal swab and saliva patient samples. *medRxiv*,  
40 2020.2008.2030.20184796, doi:10.1101/2020.08.30.20184796 (2020).
- 41 29 Lalli, M. A. *et al.* Rapid and extraction-free detection of SARS-CoV-2 from saliva  
42 with colorimetric LAMP. *medRxiv*, 2020.2005.2007.20093542,  
43 doi:10.1101/2020.05.07.20093542 (2020).
- 44 30 Ranoa, D. R. E. *et al.* Saliva-Based Molecular Testing for SARS-CoV-2 that  
45 Bypasses RNA Extraction. *bioRxiv*, 2020.2006.2018.159434,  
46 doi:10.1101/2020.06.18.159434 (2020).
- 47 31 Yang, Q. *et al.* Saliva TwoStep: An RT-LAMP saliva test for SARS-CoV-2 and its  
48 assessment in a large population. *medRxiv*, 2020.2007.2016.20150250,  
49 doi:10.1101/2020.07.16.20150250 (2020).
- 50 32 Kim, D. *et al.* The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914-921  
51 e910, doi:10.1016/j.cell.2020.04.011 (2020).

- 1 33 Lai, M. M. & Cavanagh, D. The molecular biology of coronaviruses. *Adv Virus*  
2 *Res* **48**, 1-100 (1997).
- 3 34 Li, S. *et al.* The epitope study on the SARS-CoV nucleocapsid protein. *Genomics*  
4 *Proteomics Bioinformatics* **1**, 198-206, doi:10.1016/s1672-0229(03)01025-8  
5 (2003).
- 6 35 Wu, H. Y. & Brian, D. A. Subgenomic messenger RNA amplification in  
7 coronaviruses. *Proc Natl Acad Sci U S A* **107**, 12257-12262,  
8 doi:10.1073/pnas.1000378107 (2010).
- 9 36 Zhu, J., Guo, J., Xu, Y. & Chen, X. Viral dynamics of SARS-CoV-2 in saliva from  
10 infected patients. *The Journal of infection* **81**, e48-e50,  
11 doi:10.1016/j.jinf.2020.06.059 (2020).
- 12 37 Yaffe, H. *et al.* LogSpin: a simple, economical and fast method for RNA isolation  
13 from infected or healthy plants and other eukaryotic tissues. *BMC Research*  
14 *Notes* **5**, 45, doi:10.1186/1756-0500-5-45 (2012).
- 15 38 Alekseenko, A. *et al.* Detection of SARS-CoV-2 using non-commercial RT-LAMP  
16 reagents and raw samples. *medRxiv*, 2020.2008.2022.20179507,  
17 doi:10.1101/2020.08.22.20179507 (2020).
- 18 39 Schwarzenbach, G. F., W. *Complexometric Titrations*. 2nd edn, 60-62, 260  
19 (1969).
- 20 40 Lescure, F.-X. *et al.* Clinical and virological data of the first cases of COVID-19 in  
21 Europe: a case series. *The Lancet Infectious Diseases* **20**, 697-706,  
22 doi:10.1016/S1473-3099(20)30200-0 (2020).
- 23 41 Wolfel, R. *et al.* Virological assessment of hospitalized patients with COVID-  
24 2019. *Nature* **581**, 465-469, doi:10.1038/s41586-020-2196-x (2020).
- 25 42 He, X. *et al.* Temporal dynamics in viral shedding and transmissibility of COVID-  
26 19. *Nature Medicine* **26**, 672-675, doi:10.1038/s41591-020-0869-5 (2020).
- 27 43 Moghadas, S. M. *et al.* The implications of silent transmission for the control of  
28 COVID-19 outbreaks. *Proceedings of the National Academy of Sciences* **117**,  
29 17513-17515, doi:10.1073/pnas.2008373117 (2020).
- 30 44 Broughton, J. P. *et al.* CRISPR–Cas12-based detection of SARS-CoV-2. *Nature*  
31 *Biotechnology* **38**, 870-874, doi:10.1038/s41587-020-0513-4 (2020).
- 32 45 Klein, S. *et al.* SARS-CoV-2 RNA Extraction Using Magnetic Beads for Rapid  
33 Large-Scale Testing by RT-qPCR and RT-LAMP. *Viruses* **12**, 863,  
34 doi:10.3390/v12080863 (2020).
- 35 46 Hsieh, K., Mage, P. L., Csordas, A. T., Eisenstein, M. & Tom Soh, H.  
36 Simultaneous elimination of carryover contamination and detection of DNA with  
37 uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification  
38 (UDG-LAMP). *Chemical Communications* **50**, 3747-3749,  
39 doi:10.1039/C4CC00540F (2014).
- 40 47 Ma, C. *et al.* A novel method to control carryover contamination in isothermal  
41 nucleic acid amplification. *Chemical Communications* **53**, 10696-10699,  
42 doi:10.1039/C7CC06469A (2017).
- 43 48 Helgouach, N. *et al.* EasyCOV : LAMP based rapid detection of SARS-CoV-2 in  
44 saliva. *medRxiv*, 2020.2005.2030.20117291, doi:10.1101/2020.05.30.20117291  
45 (2020).
- 46 49 Ganguli, A. *et al.* Rapid isothermal amplification and portable detection system  
47 for SARS-CoV-2. *Proceedings of the National Academy of Sciences* **117**, 22727,  
48 doi:10.1073/pnas.2014739117 (2020).

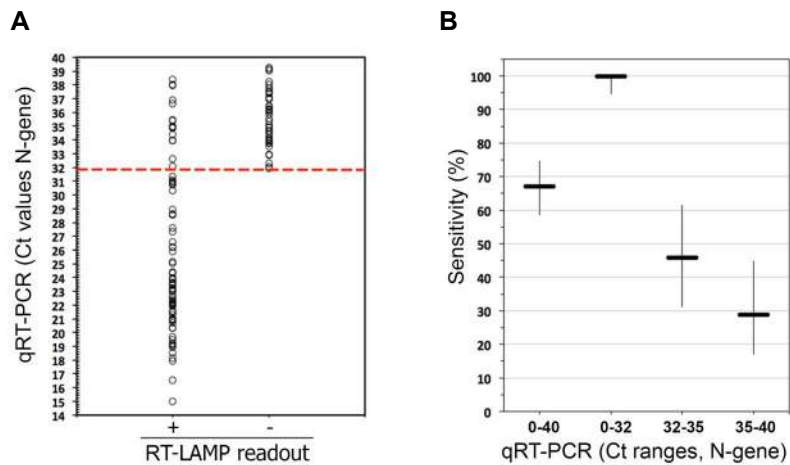
49  
50

**Figure 1**



**Fig.1 Limit of detection of the two different RT-LAMP formats and of qRT-PCR.** (A) A known number of copies of *in vitro* transcribed (IVT) viral RNA (N-gene) were amplified and detected by colorimetric RT-LAMP using the (i) WarmStart Colorimetric LAMP 2x Master Mix (New England Biolabs) or (ii) the separate components (enzymes purchased individually and an in-house-made colorimetric buffer). The reactions were incubated at 65°C for 30 minutes. (B) 10 µl of the RT-LAMP reaction were resolved in an agarose gel (2%) electrophoresis. The ladder pattern corresponds to the expected LAMP amplification pattern. (C) Limit of detection of ten replicates of the two test formats. (D) Standard curve generated by plotting the number of IVT RNA copies (x-axis) vs. the mean of the corresponding qRT-PCR threshold cycle (Ct) value (y-axis) of three independent experiments.

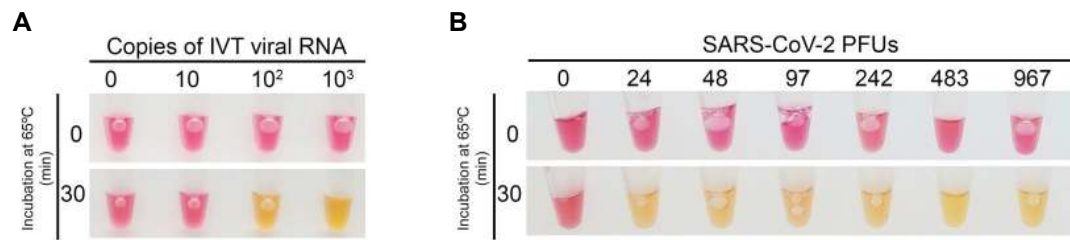
**Figure 2**



**Fig 2. Detection of SARS-CoV-2 in NP samples using RT-LAMP.** (A) Comparison of RT-LAMP and qRT-PCR results. The Ct values (qRT-PCR results) of 126 COVID-19 positive patients (y-axis) were compared to the RT-LAMP readout (x-axis) taken after 30 minutes of incubation at 65°C (positive, + /yellow; negative, - /pink). The dotted red line indicates the Ct below, which there is 100% agreement between RT-LAMP and qRT-PCR. (B) Sensitivity of the RT-LAMP assay across different ranges of Ct values (which reflect different viral loads). The thicker horizontal lines indicate the specificity calculated for the indicated Ct range (according to the data of panel A and Table 1). The vertical lines indicate the corresponding 95% confidence intervals.

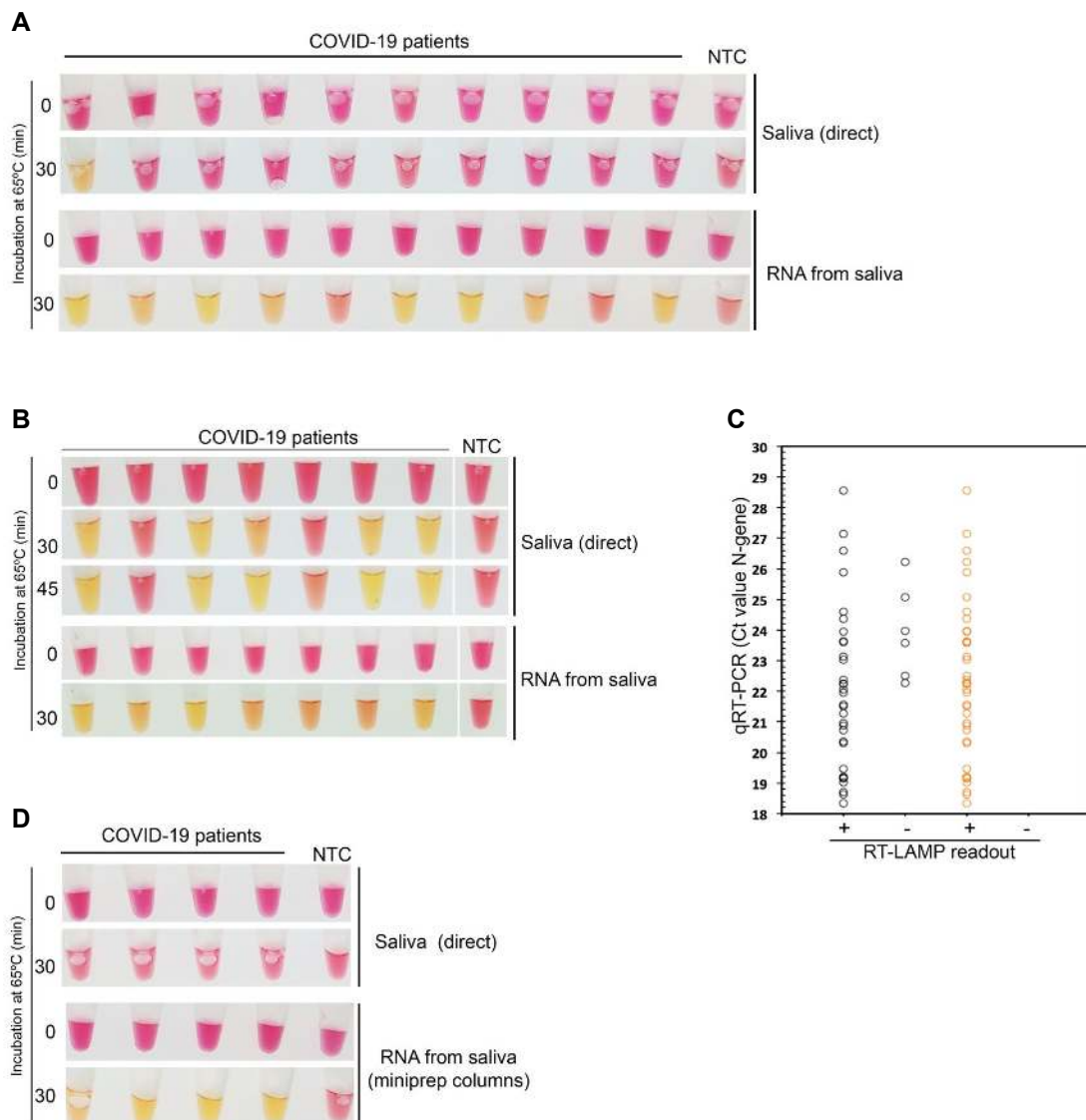


**Figure 3**



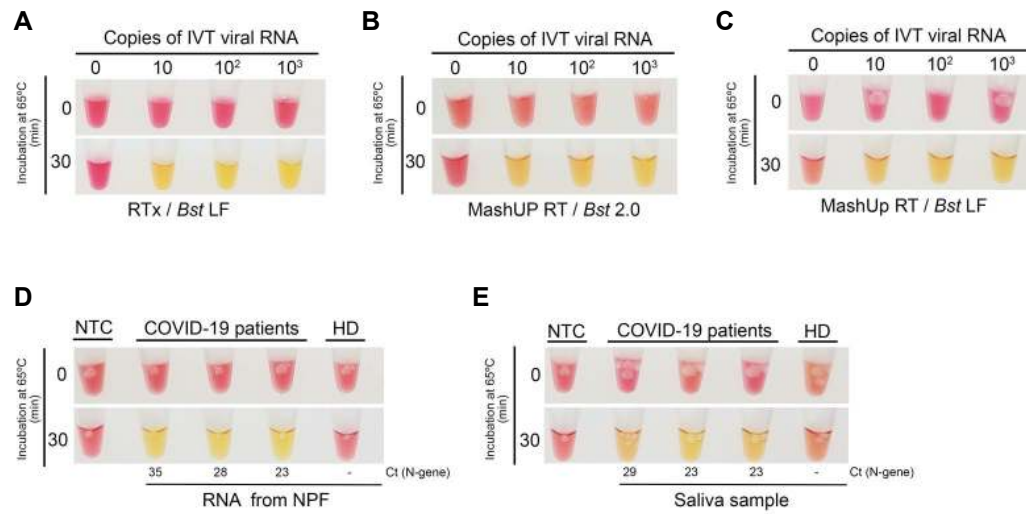
**Fig.3 Limit of detection of the saliva RT-LAMP assay.** Spike-in experiments of a healthy donor saliva with (A) tenfold dilutions of *in vitro* transcribed (IVT) viral RNA (N-gene) and (B) *in vitro* propagated SARS-CoV-2 virions. Saliva samples were processed as described in Material and Methods and 2  $\mu$ L were analyzed by colorimetric RT-LAMP. PFUs – plaque forming units.

**Figure 4**



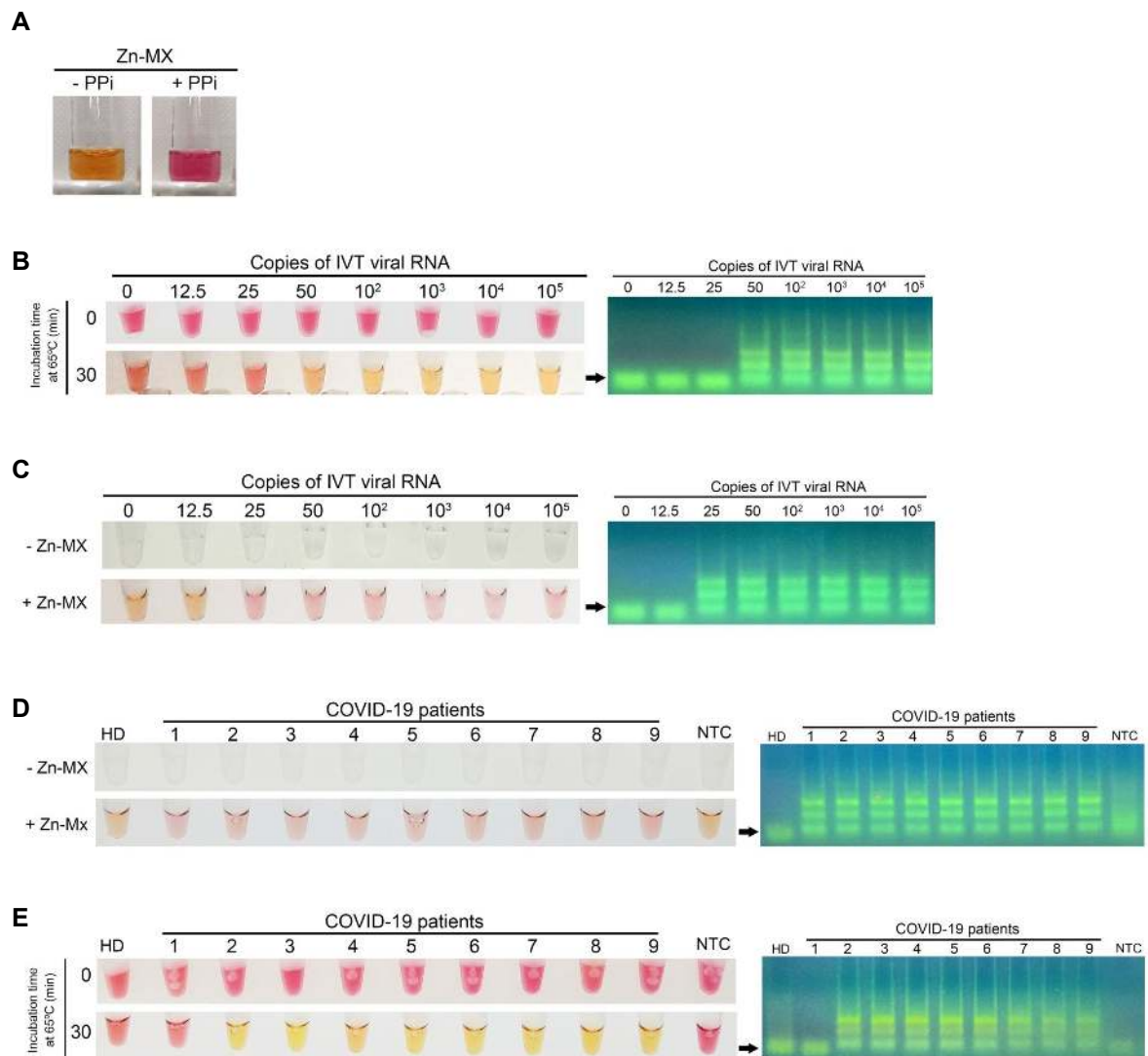
**Fig.4 Detection of SARS-CoV-2 in saliva samples using RT-LAMP.** RT-LAMP analysis of saliva samples of confirmed COVID-19 patients who (A) induced or not (B) salivation before sample collection. (C) Comparison of RT-LAMP and qRT-PCR results. The Ct values (qRT-PCR results) of 39 COVID-19 positive patients (y-axis) were compared to the RT-LAMP readout of the matched saliva samples (x-axis), after 30 minutes of incubation at 65°C (positive, + /yellow; negative, - /pink). Black circles – direct saliva; orange circles – RNA extracted from saliva. (D) RNAs from the saliva of false negative samples (as determined by the direct saliva test) were extracted using plasmid DNA miniprep columns (ZR Plasmid Miniprep-Classic Kit, Zymo Research) and re-analyzed. NTC – No template control.

**Figure 5**



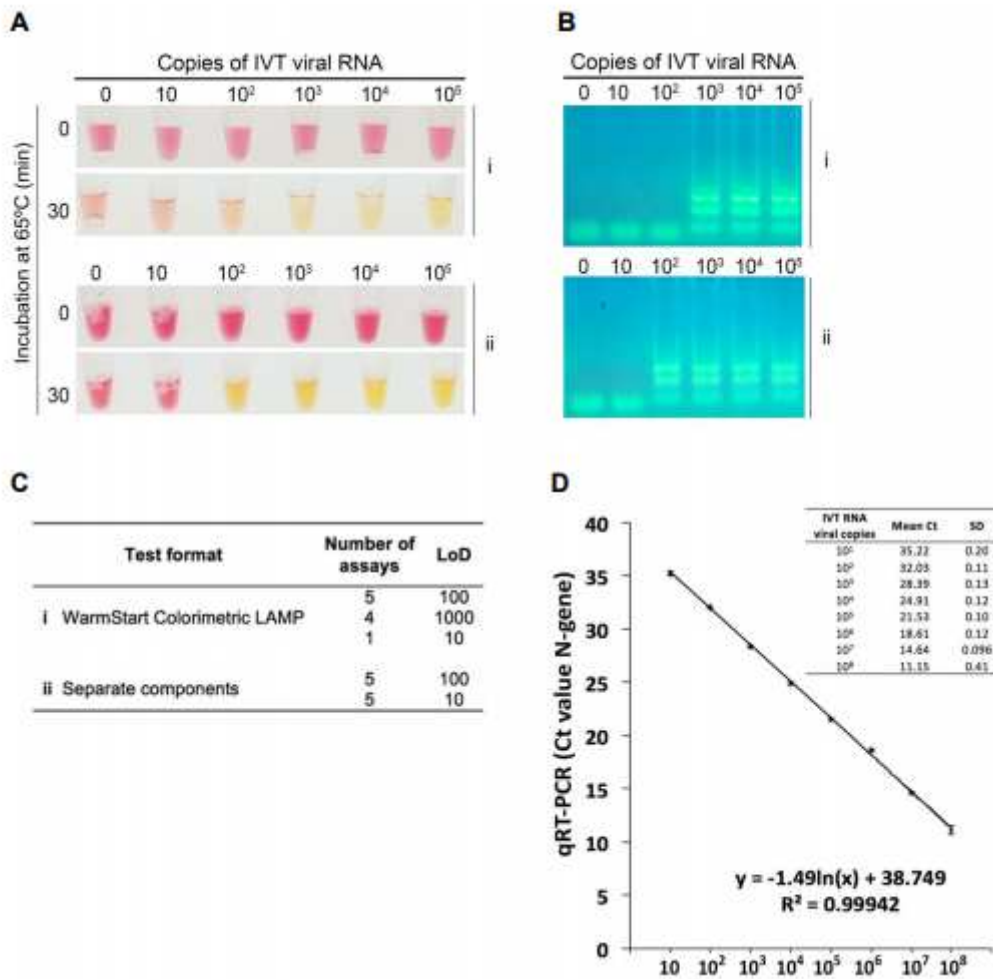
**Fig.5 Analytical sensitivity of an in-house-made colorimetric RT-LAMP assay.** Tenfold dilutions of *in vitro* transcribed (IVT) viral RNA (N-gene) were amplified *via* RT-LAMP and detected using a colorimetric buffer together with (A) RTx (New England Biolabs) and *Bst* LF (homemade), (B) MashUP RT (homemade) and *Bst* 2.0 (New England Biolabs) or (C) MashUP RT (homemade) and *Bst* LF (homemade). The in-house-made setup was next used to detect SARS-CoV-2 sequences in (D) RNAs extracted from the NP fluid (NPF) and (E) saliva samples of COVID-19 positive patients. The reactions were incubated at 65°C for 30 minutes. NTC – No template control, HD – Healthy donor.

**Figure 6**



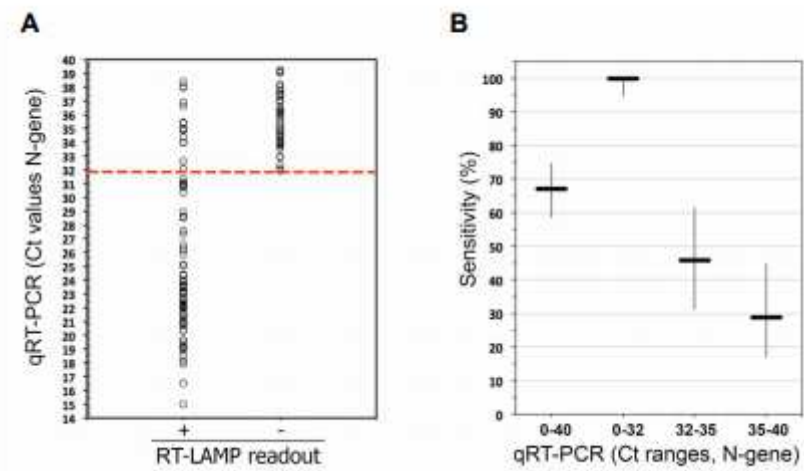
**Fig.6 Alternative colorimetric detection based on the complex murexide-zinc.** (A) A strong color change from yellow to pink is observed when pyrophosphate (PPI) is added to a solution containing Zn-MX. Tenfold dilutions of *in vitro* transcribed (IVT) viral IVT RNA (N-gene) were amplified *via* RT-LAMP and detected using phenol red (B) or Zn-MX (C). Amplification was confirmed by agarose gel electrophoresis (AGE). Saliva samples of a healthy donor (HD) and of nine COVID-19 patients were analyzed by RT-LAMP followed by detection with Zn-MX (D) or phenol red (E) and amplification was confirmed by AGE.

# Figures



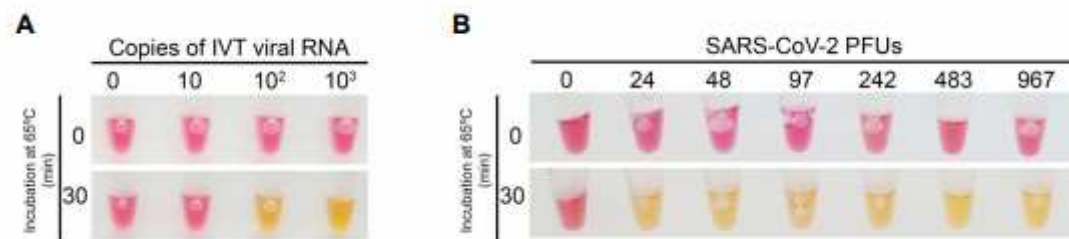
**Figure 1**

Limit of detection of the two different RT-LAMP formats and of qRT-PCR. (A) A known number of copies of in vitro transcribed (IVT) viral RNA (N-gene) were amplified and detected by colorimetric RT-LAMP using the (i) WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs) or (ii) the separate components (enzymes purchased individually and an in-house-made colorimetric buffer). The reactions were incubated at 65°C for 30 minutes. (B) 10 µl of the RT-LAMP reaction were resolved in an agarose gel (2%) electrophoresis. The ladder pattern corresponds to the expected LAMP amplification pattern. (C) Limit of detection of ten replicates of the two test formats. (D) Standard curve generated by plotting the number of IVT RNA copies (x-axis) vs. the mean of the corresponding qRT-PCR threshold cycle (Ct) value (y-axis) of three independent experiments.



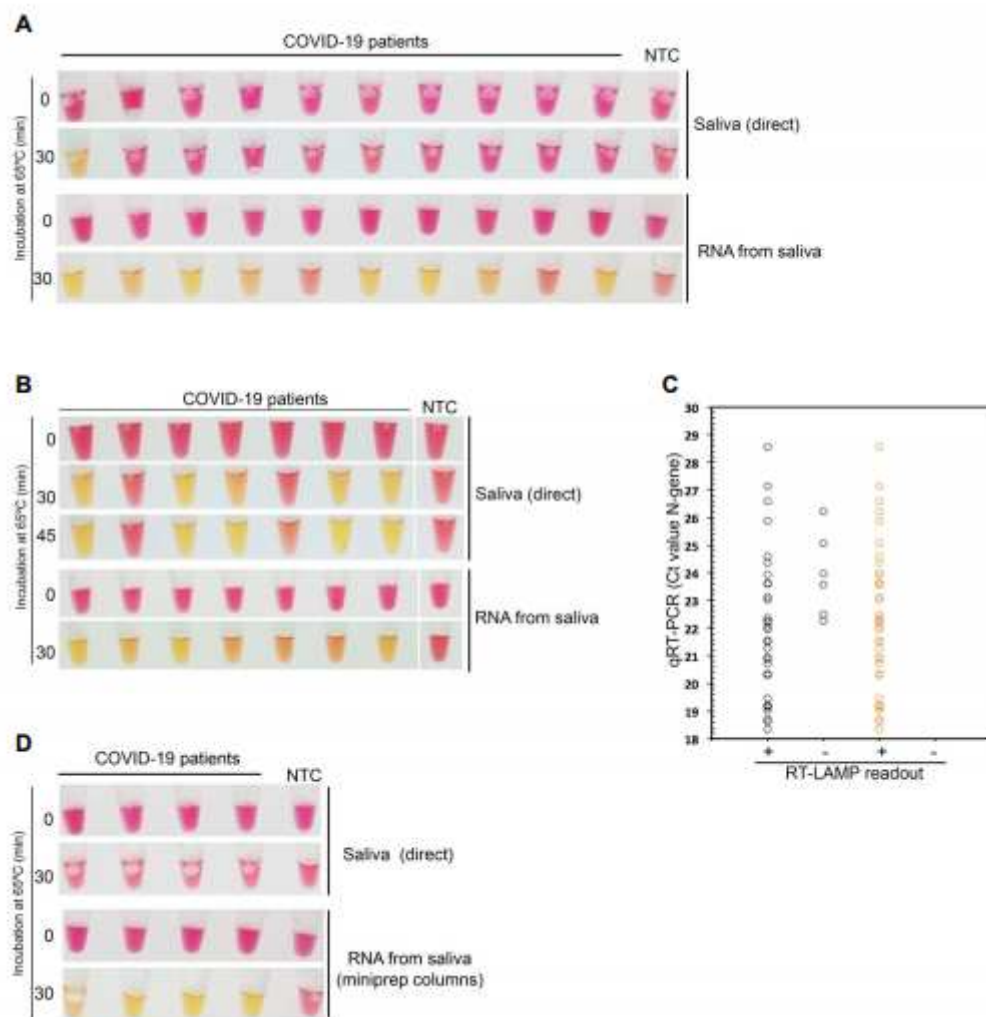
**Figure 2**

Detection of SARS-CoV-2 in NP samples using RT-LAMP. (A) Comparison of RT-LAMP and qRT-PCR results. The Ct values (qRT-PCR results) of 126 COVID-19 positive patients (y-axis) were compared to the RT-LAMP readout (x-axis) taken after 30 minutes of incubation at 65°C (positive, + /yellow; negative, - /pink). The dotted red line indicates the Ct below, which there is 100% agreement between RT-LAMP and qRT-PCR. (B) Sensitivity of the RT-LAMP assay across different ranges of Ct values (which reflect different viral loads). The thicker horizontal lines indicate the specificity calculated for the indicated Ct range (according to the data of panel A and Table 1). The vertical lines indicate the corresponding 95% confidence intervals.



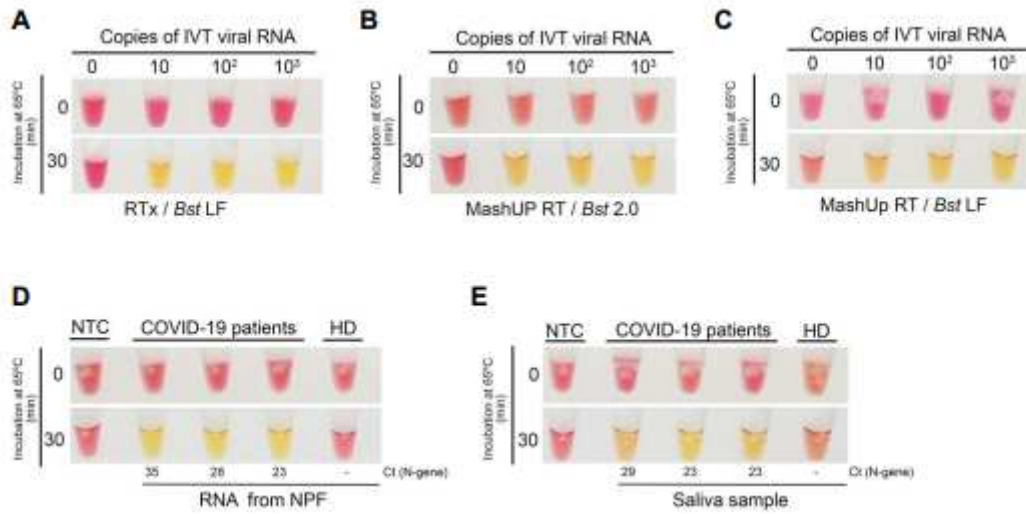
**Figure 3**

Limit of detection of the saliva RT-LAMP assay. Spike-in experiments of a healthy donor saliva with (A) tenfold dilutions of in vitro transcribed (IVT) viral RNA (N-gene) and (B) in vitro propagated SARS-CoV-2 virions. Saliva samples were processed as described in Material and Methods and 2 µL were analyzed by colorimetric RT-LAMP. PFUs – plaque forming units.



**Figure 4**

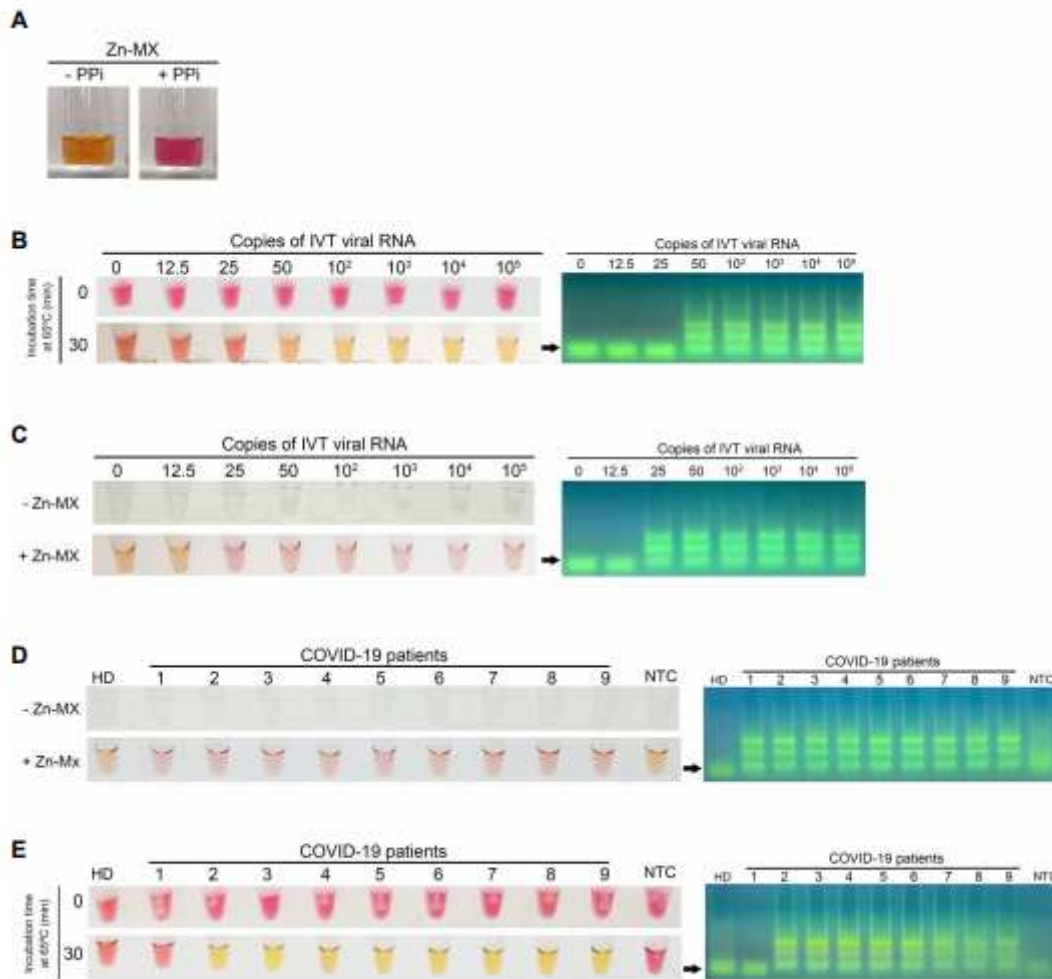
Detection of SARS-CoV-2 in saliva samples using RT-LAMP. RT-LAMP analysis of saliva samples of confirmed COVID-19 patients who (A) induced or not (B) salivation before sample collection. (C) Comparison of RT-LAMP and qRT-PCR results. The Ct values (qRT-PCR results) of 39 COVID-19 positive patients (y-axis) were compared to the RT-LAMP readout of the matched saliva samples (x-axis), after 30 minutes of incubation at 65°C (positive, + /yellow; negative, - /pink). Black circles – direct saliva; orange circles – RNA extracted from saliva. (D) RNAs from the saliva of false negative samples (as determined by the direct saliva test) were extracted using plasmid DNA miniprep columns (ZR Plasmid Miniprep-Classic Kit, Zymo Research) and re-analyzed. NTC – No template control.



**Figure 5**

Analytical sensitivity of an in-house-made colorimetric RT-LAMP assay. Tenfold dilutions of in vitro transcribed (IVT) viral RNA (N-gene) were amplified via RT-LAMP and detected using a colorimetric buffer together with (A) RTx (New England Biolabs) and Bst LF (homemade), (B) MashUP RT (homemade) and Bst 2.0 (New England Biolabs) or (C) MashUP RT (homemade) and Bst LF (homemade). The in-house-made setup was next used to detect SARS-CoV-2 sequences in (D) RNAs extracted from the NP fluid (NPF) and (E) saliva samples of COVID-19 positive patients. The reactions were incubated at 65°C for 30 minutes. NTC – No template control, HD – Healthy donor.





**Figure 6**

Alternative colorimetric detection based on the complex murexide-zinc. (A) A strong color change from yellow to pink is observed when pyrophosphate (PPI) is added to a solution containing Zn-MX. Tenfold dilutions of in vitro transcribed (IVT) viral RNA (Ngene) were amplified via RT-LAMP and detected using phenol red (B) or Zn-MX (C). Amplification was confirmed by agarose gel electrophoresis (AGE). Saliva samples of a healthy donor (HD) and of nine COVID-19 patients were analyzed by RT-LAMP followed by detection with Zn-MX (D) or phenol red (E) and amplification was confirmed by AGE.

## Supplementary Files

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

- [SupplementaryMaterial.pdf](#)