Supplementary Information

Microfluidic Point-Of-Care Device for Detection of Early Strains and B.1.1.7 Variant of SARS-CoV-2 Virus

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Contents

A

N-gene

Primer	Sequence
F3	GTT CCT CAT CAC GTA GTC G
B3	GTT TGG CCT TGT TGT TGT T
FIP	GCC AGC CAT TCT AGC AGG AG- CAA CAG TTA AGA AAT TCA ACT CC
BIP	GAT GCT GCT CTT GCT TTG CT- ACC AGA CAT TTT GCT CTC AA
Loop B	GCT GCT TGA CAG ATT GAA CCA G

B

S-gene

Primer	Sequence
F3	GGT GTT TAT TAC CCT GAC AAA G
B3	GTA CCA AAA ATC CAG CCT C
FIP	TGG AAC CAA GTA ACA TTG GAA AAG A- TTT TCA GAT CCT CAG TTT TAC ATT C
BIP	CTC TGG GAC CAA TGG TAC TAA GAG- GAC TTC TCA GTG GAA GCA
Loop B	AAC CCT GTC CTA CCA TTT AAT GAT G

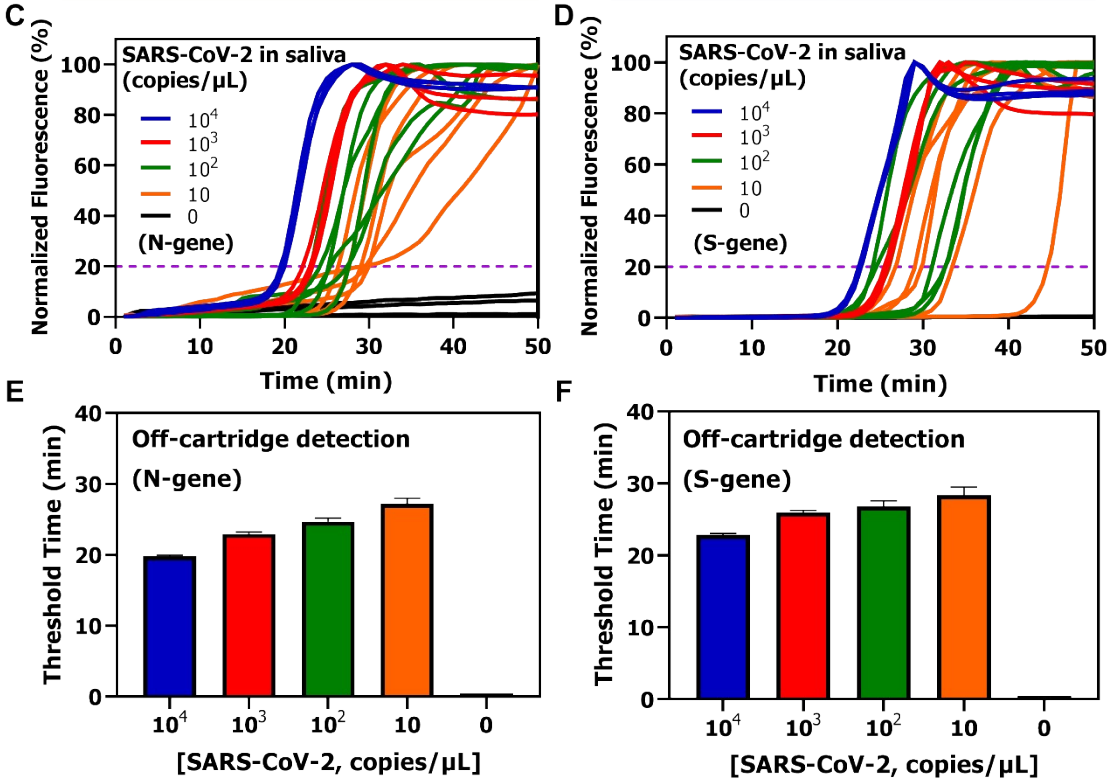


Figure S1. Characterization of the detection limit of the off-cartridge RT-LAMP assay for the detection of the SARS-CoV-2 virus spiked in saliva. RT-LAMP primers sequences for (A) N-gene and (B) S-gene. Normalized fluorescence data (n = 3-6) for SARS-CoV-2 detection, (C) N-gene and (D) S-gene, from spiked saliva samples. For the concentrations of 10^2 and 10 copies/µL, n = 6 replicates were tested for a reliable LOD characterization. Amplification threshold time was calculated at 20% of the fluorescence signal, for (E) N-gene and (F) S-gene.

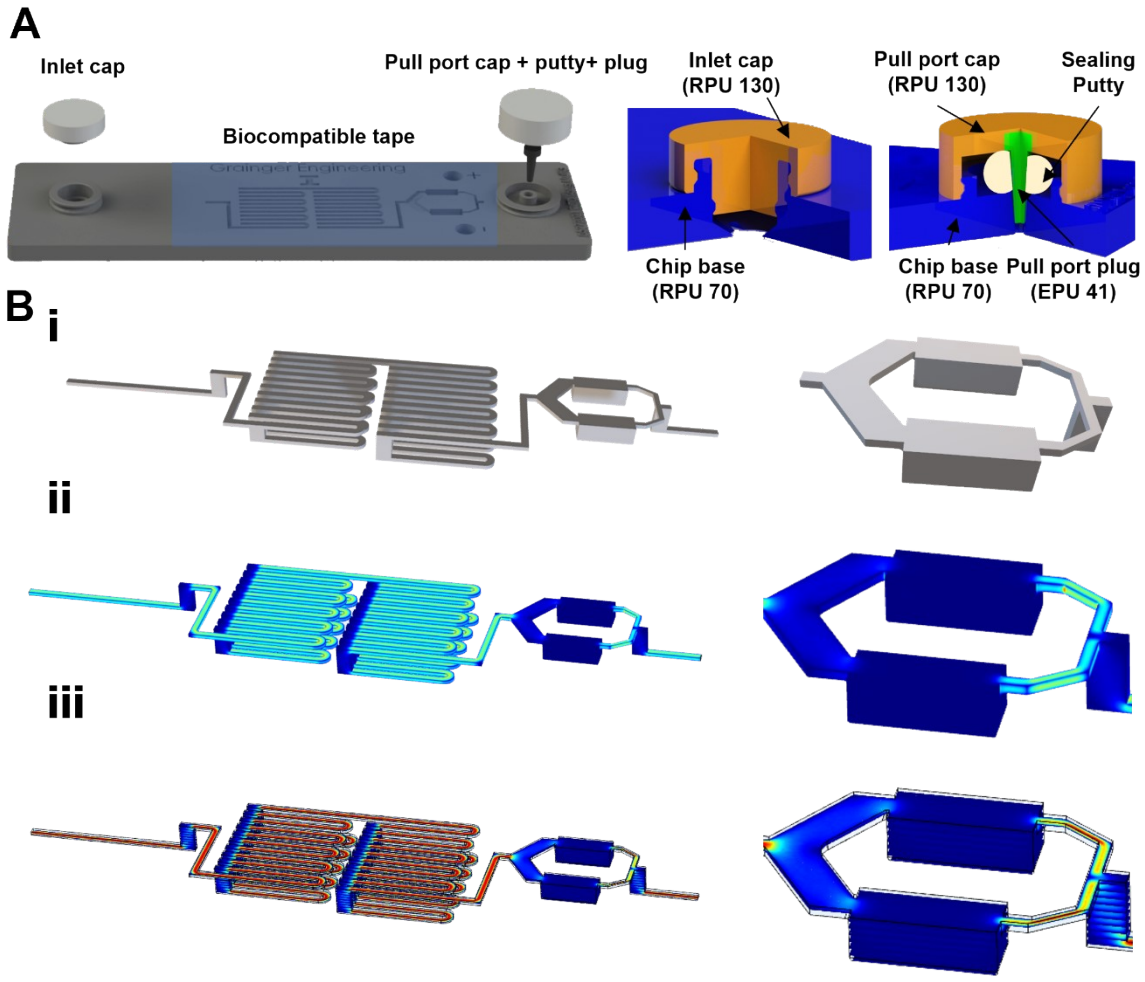


Figure S2. Additively manufactured cartridge and flow details. (A) Cartridge sealing mechanisms. Precise snap-fit caps were fabricated to seal the inlet and pull ports. Additionally, a plug was fabricated from a soft material to stop any fluid flow through the pull port. (B) (i) Extracted channel geometry; (ii) Relative shear rate of fluid flow through the channels; (iii) Relative fluid velocity of flow in channels. The relatively high shear rate and fluid velocity at the end of the detection regions allow the two regions to be filled sequentially.

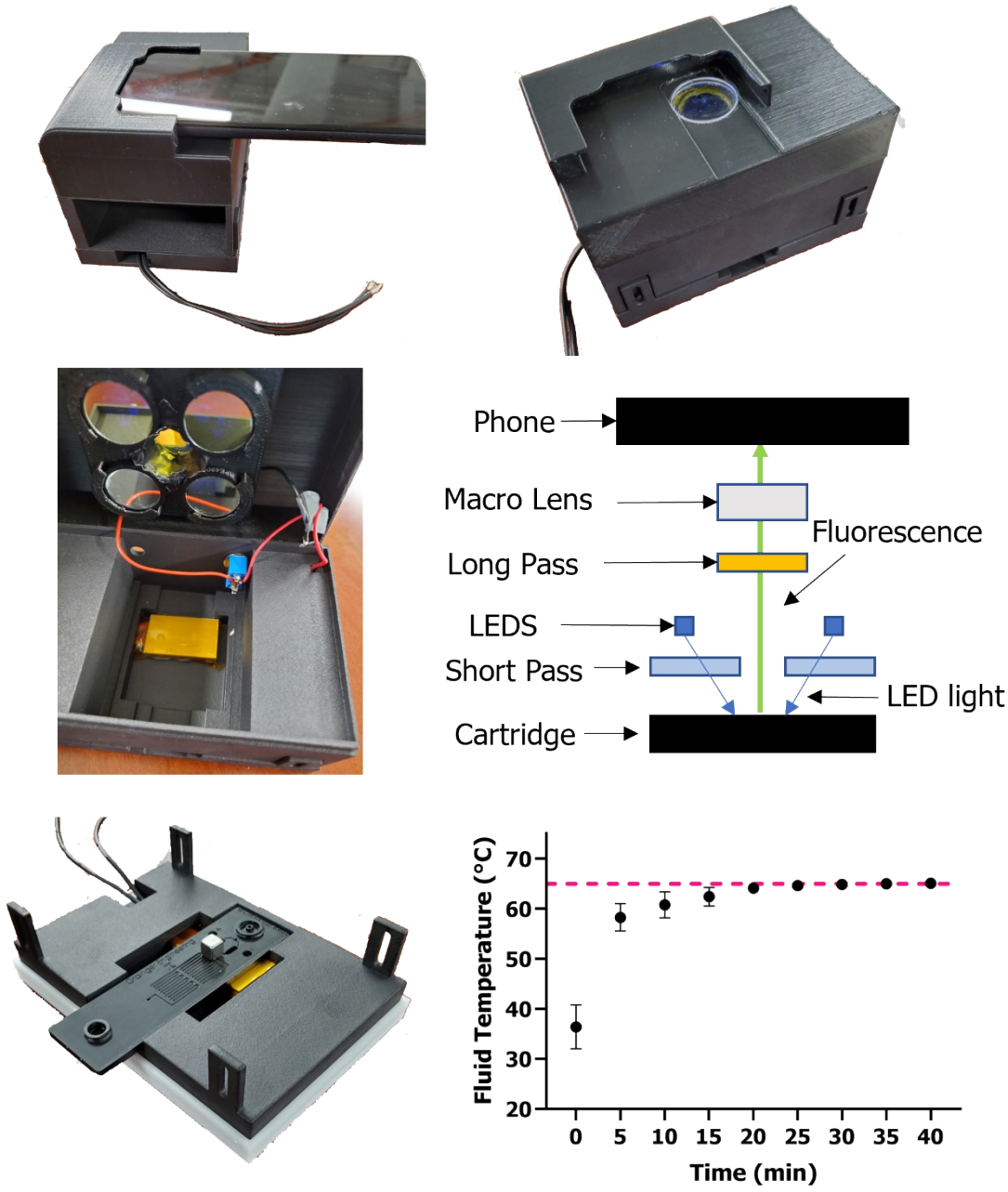


Figure S3. Point-of-care reader. (A) The reader body is 3D printed and houses the on-board heater, magnetic clamping system and optical array. (B) The optical array consists of a macro lens, a long pass filter, LEDs, and short pass filters. The LED light is passed through the short pass filter to illuminate the detection region, the fluorescence is passed through a long pass filter and a macro lens to the smartphone which takes the images throughout the amplification process. (C) Temperature calibration using the internal heater located at the bottom of the cradle.

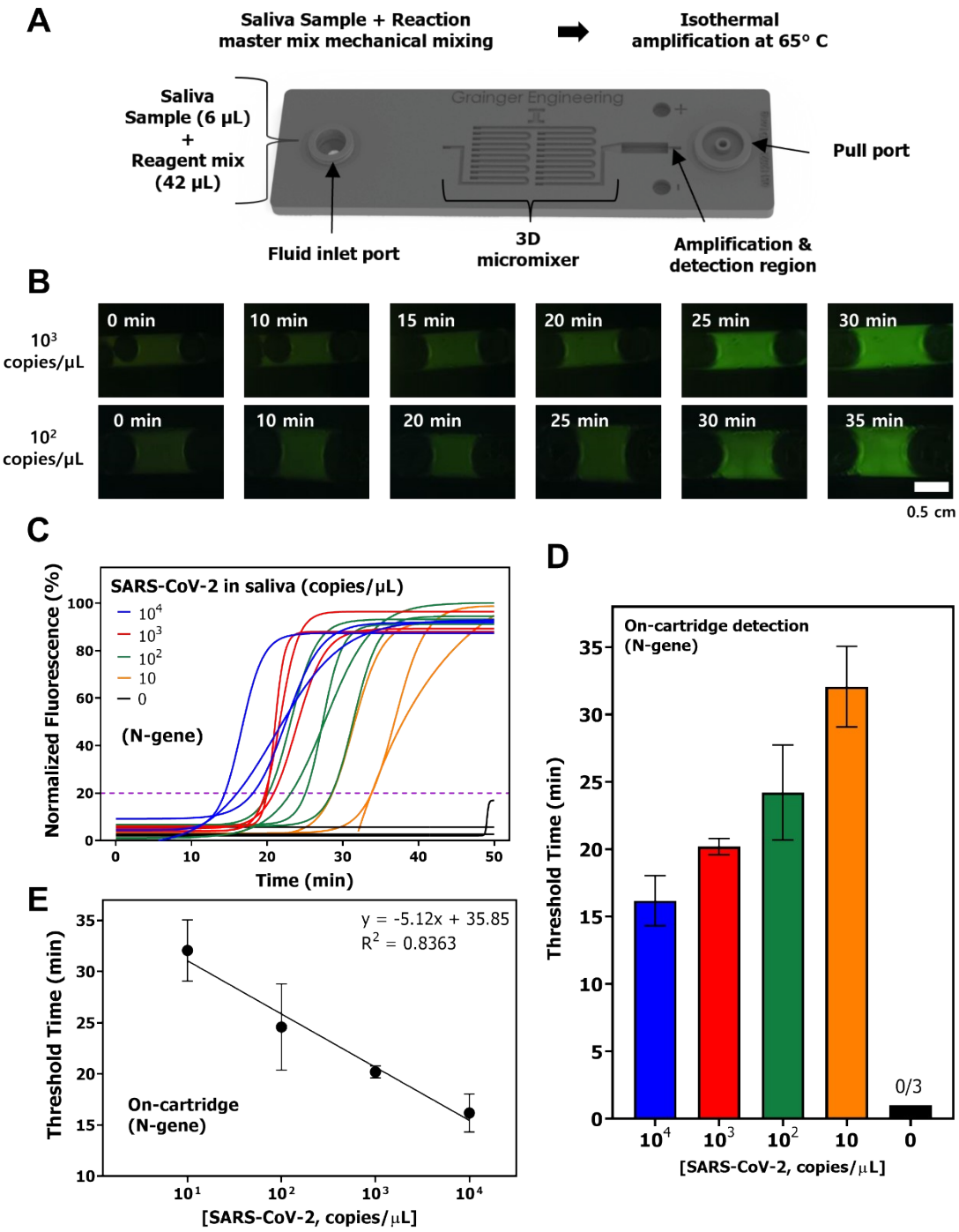


Figure S4. On-cartridge (single detection reservoir) LOD characterization of the RT-LAMP assay for the detection of SARS-CoV-2 virus spiked in saliva. (A) Schematic of a cartridge with a single detection reservoir. No primer dehydration step performed (primers were included in the reaction mix). (B) Fluorescence images of the real-time RT-LAMP reaction for single gene detection (N-gene) of SARS-CoV-2 on the additively manufactured cartridge. The reaction was conducted at 65°C for 50 min. Scale bar = 0.5 cm. (C) Normalized fluorescence data (n = 3) for SARS-CoV-2 detection (N-gene) from spiked saliva samples. (D) Amplification threshold time calculated at 20% of the fluorescence signal. (E) Calibration curves for detection of SARS-CoV-2 virus (N-gene) spiked in saliva.

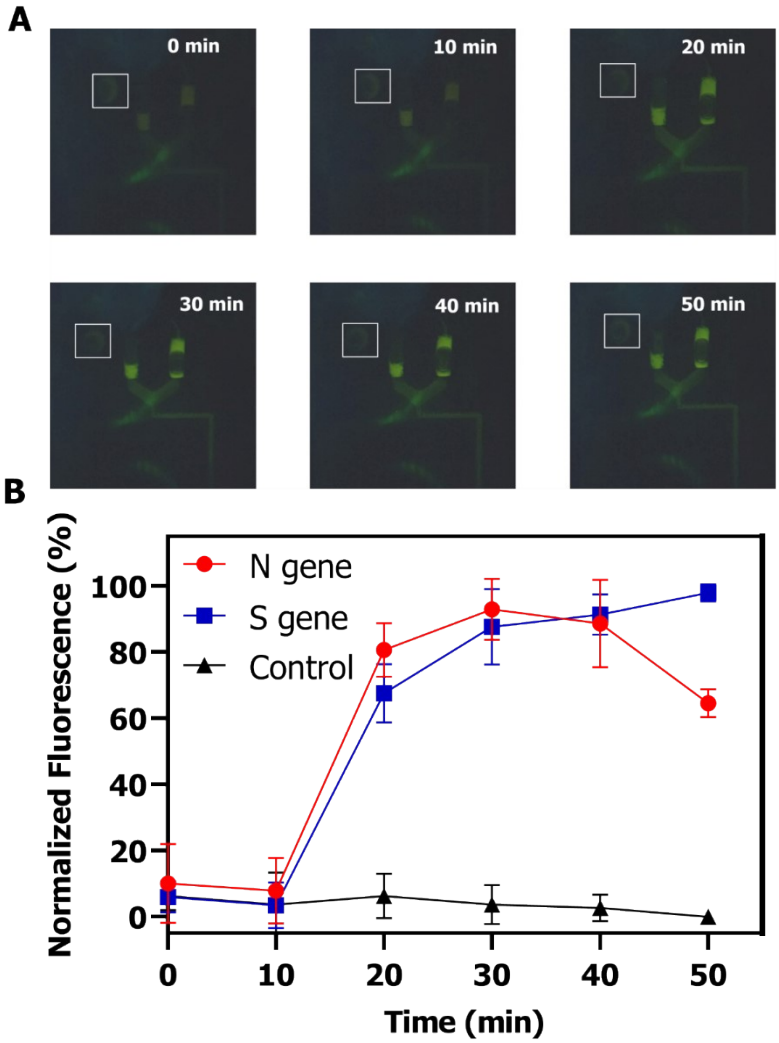


Figure S5. On-cartridge SARS-CoV-2 detection together with the negative control region. (A) Fluorescence images of real-time RT-LAMP reaction from the multiplexed POC reaction simultaneously together with control region. Negative control region was highlighted with the white box and did not show significant fluorescence increment, whereas fluorescence of other two regions (N-gene at left side and S-gene at right side) increased significantly with 10^3 copies/μL of target viral concentration. (B) Normalized fluorescence curves were plotted with 10 minutes of intervals, confirming that the cartridges can be used for the simultaneous detection of the negative control with multiplexed detection reservoirs.

A

Sample ID	RT-PCR (Ct value)			RT-LAMP (amplification time, min)	
	N-gene	ORF1ab-gene	S-gene	N-gene	S-gene
S1	30.37 ± 0.52	31.37 ± 0.30	32.75 ± 0.63	29.56 (1/6)	32.35 (1/3)
S2	26.61 ± 0.12	27.57 ± 0.28	28.46 ± 0.18	21.35 ± 0.67	22.94 ± 0.77
S3	33.22 ± 0.15	34.72 (1/3)	35.31 ± 1.21 (2/3)	---	---
S4	21.97 ± 0.14	23.45 ± 0.03	23.12 ± 0.10	22.27 ± 0.28	22.23 ± 1.80
S5	29.14 ± 0.32	32.92 ± 0.40	32.26 ± 0.42	20.39 ± 0.66	20.42 ± 0.84
S6	25.66 ± 0.10	25.81 ± 0.21	26.60 ± 0.25	18.66 ± 0.33	20.55 ± 0.50
S7	27.38 ± 0.06	30.00 ± 0.08	30.42 ± 0.13	23.69 ± 4.04	27.66 ± 0.21
S8	20.26 ± 0.16	22.67 ± 0.31	22.59 ± 0.36	21.15 ± 0.30	21.80 ± 0.69
S9	36.09 ± 1.37	36.17 ± 0.30	---	22.99 ± 2.78	27.25 ± 0.40
S10	26.35 ± 0.14	28.35 ± 0.01	29.07 ± 0.51	30.17 ± 3.28	31.74 ± 1.19
S11	32.29 ± 0.28	33.08 ± 0.26	33.96 ± 0.48	30.93 ± 0.17 (2/6)	29.47 (1/3)
S12	---	---	---	---	---
S13	---	---	---	---	---
S14	---	---	---	---	---
S15	36.23 (1/3)	---	37.03 (1/3)	---	---
S16	37.10 (1/3)	---	---	---	---
S17	35.17 (1/3)	---	---	---	---
S18	---	---	---	---	---

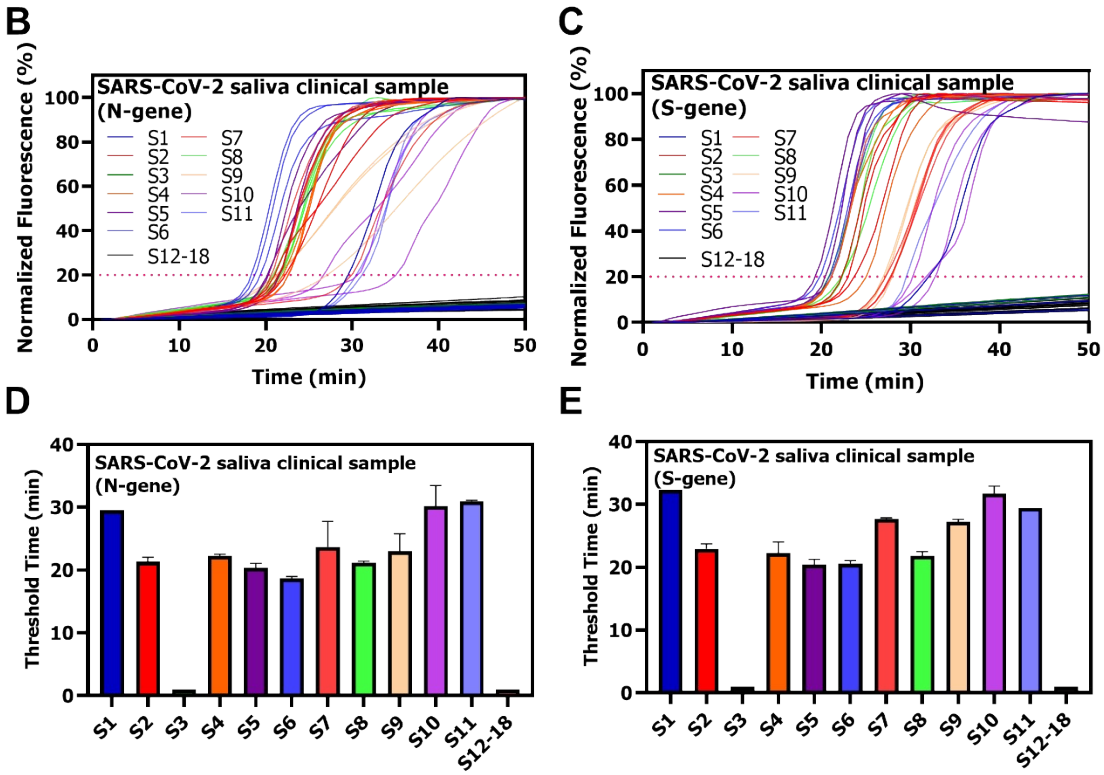


Figure S6. Off-cartridge detection of SARS-CoV-2 early strains from clinical saliva samples. (A) Summary of the results for 18 clinical samples analyzed by the RT-PCR assay and RT-LAMP assay. Normalized fluorescence data (n = 3) for the detection of the clinical saliva samples with two target-specific primer sets, (B) N-gene and (C) S-gene. Amplification threshold time calculated from 20% of fluorescence for (D) N-gene and (E) S-gene.

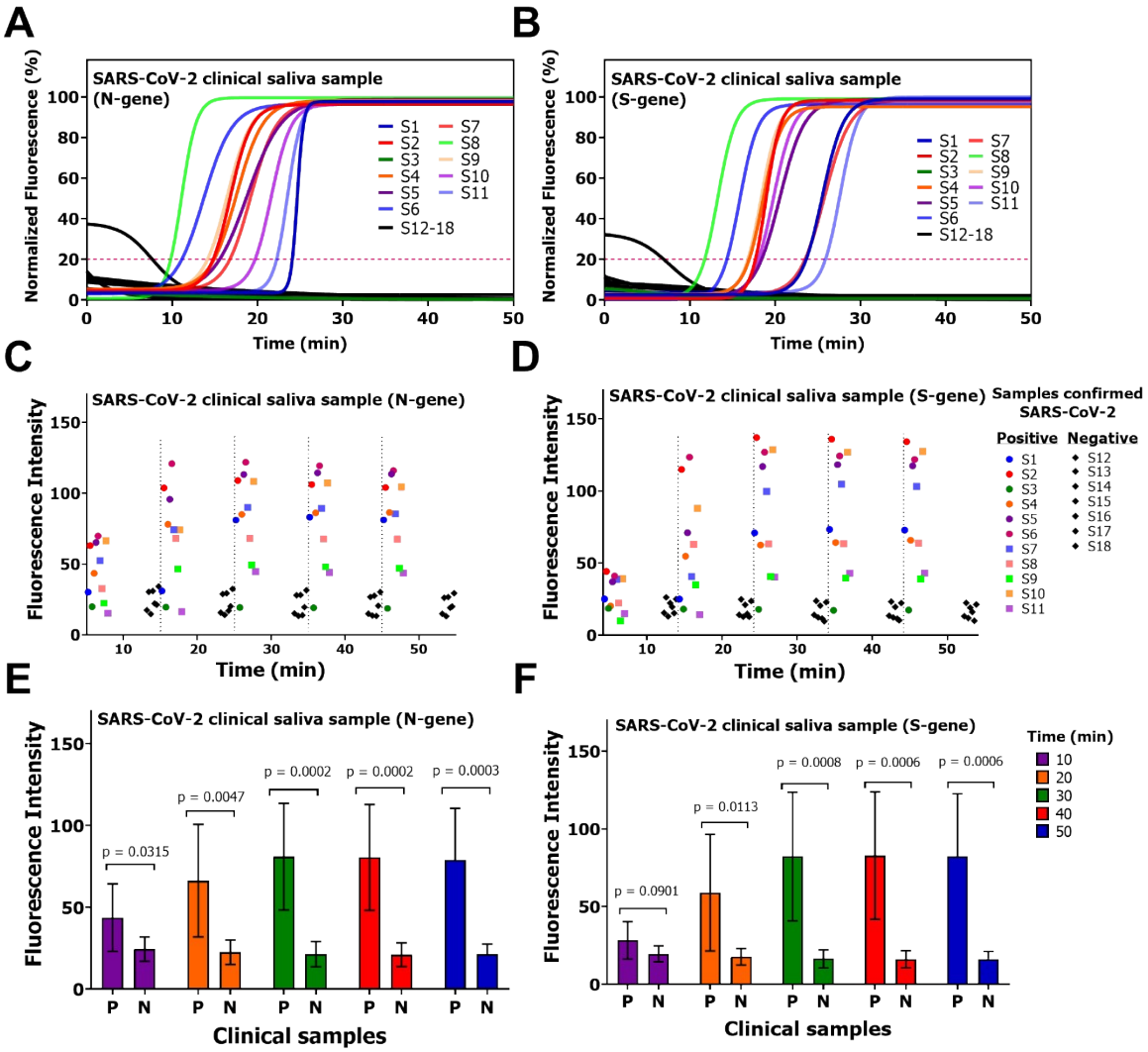


Figure S7. On-cartridge real-time multiplexed detection of 18 SARS-CoV-2 early strains clinical saliva samples. Normalized fluorescence amplification curves for detection of (A) N-gene and (B) S-gene. Fluorescence intensities (C) N-gene and (D) S-gene, with five different time points. Statistical comparison analysis between positive and negative clinical samples for (E) N-gene and (F) S-gene, according to time. Based on the t-tests performed, we determined that even though we ran the RT-LAMP reaction for 50 min., it could reduce to 30 minutes of amplification time.

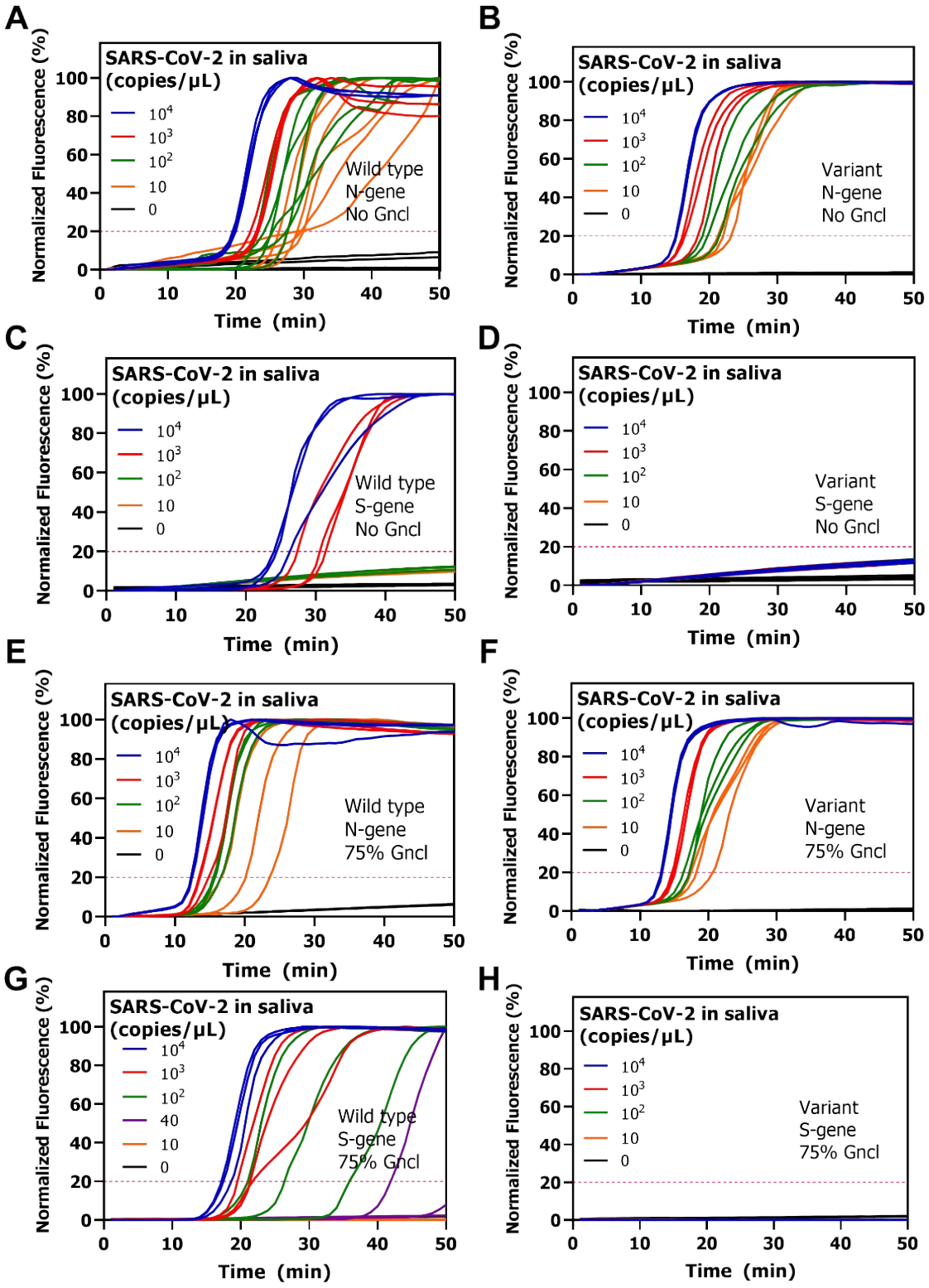


Figure S8. (A-D) Off-cartridge RT-LAMP assay for SARS-CoV-2 virus detection (B.1.1.7 variant and SARS-CoV-2 early strains) spiked in saliva using the reaction mix without GnCl. Normalized fluorescence amplification curves (n = 3) for (A) SARS-CoV-2 early strains, N-gene; (B) B.1.1.7 variant, N-gene; (C) SARS-CoV-2 early strains, S-gene; and (D) B.1.1.7 variant, S-gene. (E-H) Off-cartridge RT-LAMP assay for SARS-CoV-2 virus detection (B.1.1.7 variant and SARS-CoV-2 early strains) spiked in saliva using the reaction mix with 11mM GnCl. Normalized fluorescence amplification curves (n = 3) for (E) SARS-CoV-2 early strains, N-gene; (F) B.1.1.7 variant, N-gene; (G) SARS-CoV-2 early strains, S-gene; and (H) B.1.1.7 variant, S-gene.

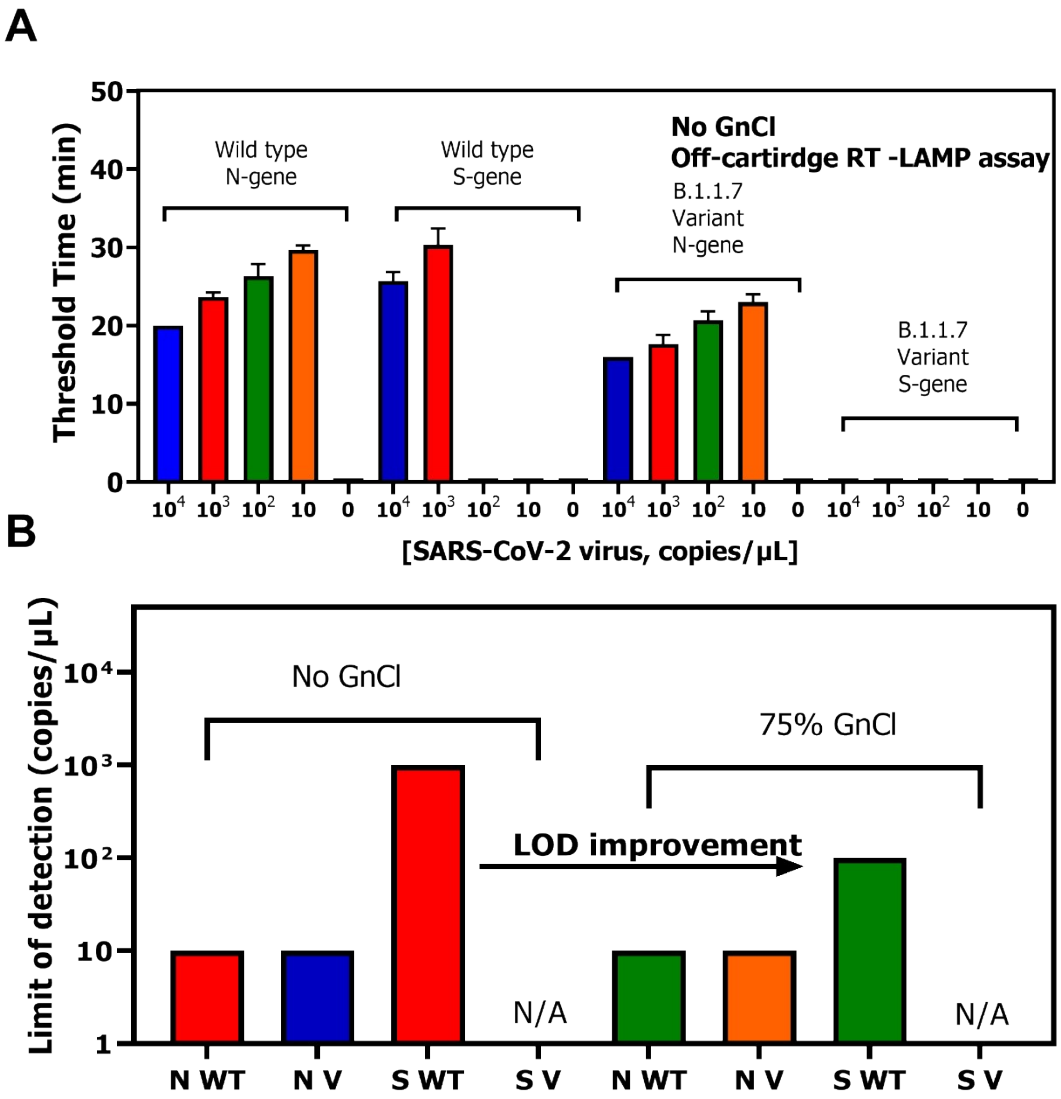
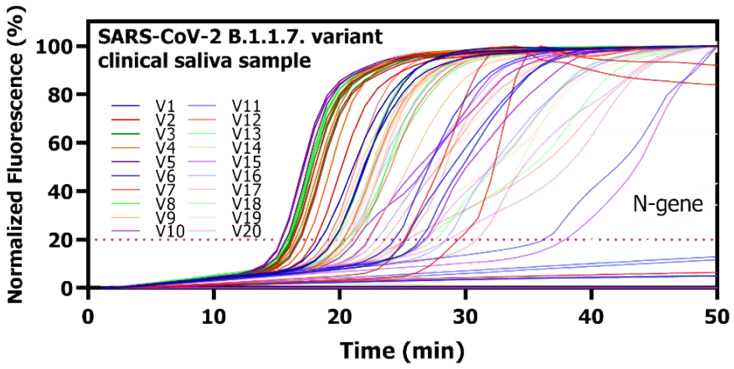


Figure S9. (A) Off-cartridge LOD characterization of the RT-LAMP assay without GnCl for the differentiation of two different SARS-CoV-2 virus (B.1.1.7 variant and SARS-CoV-2 early strains) in saliva. (B) Comparison of the off-cartridge detection assay without and with 11mM GnCl in the RT-LAMP reaction mix. LOD of the S-gene specific to detect the SGTF against the SARS-CoV-2 early strains improved 10X fold without affecting LOD of other assays. N WT is N-gene from the SARS-CoV-2 early strains. N V is N-gene from the B.1.1.7 variant. S WT is S-gene from the SARS-CoV-2 early strains.

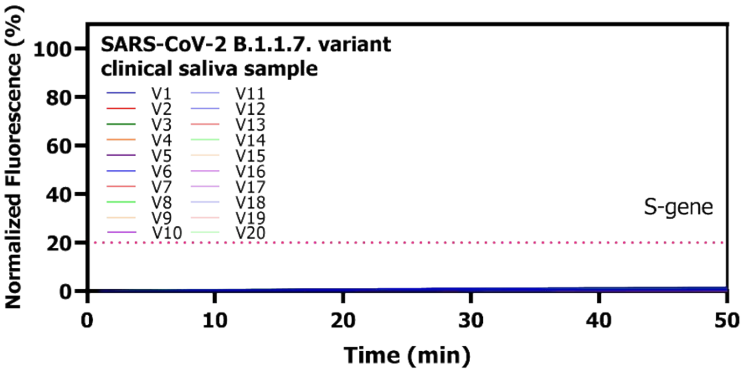
A

Sample ID	RT-PCR assay (CT value)			RT-LAMP assay (amplification time ,min)	
	N-gene	ORF1ab-gene	S-gene	N-gene	S-gene
V1	24.27	25	---	18.96 ± 0.22	---
V2	20.25	21.2	---	16.65 ± 0.41	---
V3	20.77	21.82	---	16.03 ± 0.32	---
V4	22.01	22.53	---	16.49 ± 0.40	---
V5	21.5	22.52	---	15.24 ± 0.16	---
V6	18.44	20.4	---	24.57 ± 0.59	---
V7	21.31	23.34	---	27.76 ± 1.76 (2/3)	---
V8	20.63	21.63	---	15.64 ± 0.35	---
V9	25.64	27.2	---	20.20 ± 0.36	---
V10	28.85	30.73	---	22.48 ± 0.93	---
V11	35.88	34.19	---	40.08 (1/6)	---
V12	24.13	24.91	---	19.70 ± 0.31	---
V13	24.04	25.3	---	20.20 ± 0.26	---
V14	25.32	25.81	---	19.97 ± 0.16	---
V15	34.33	34.71	---	42.98 (1/6)	---
V16	32.36	31.33	---	21.77 ± 0.87	---
V17	27.7	28.37	---	23.84 ± 1.18	---
V18	30.42	30.77	---	21.16 ± 1.78	---
V19	30.79	31.02	---	25.17 ± 1.03	---
V20	33.52	35.64	---	27.89 ± 0.58	---

B



C



D

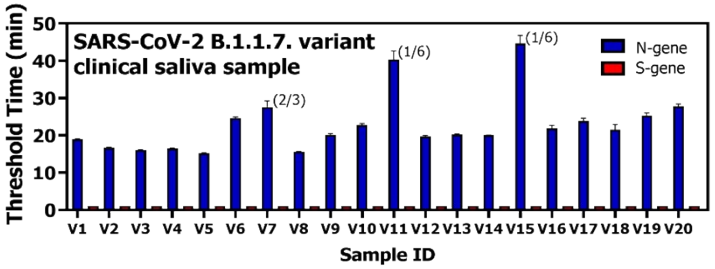


Figure S10. Off-cartridge detection of variant saliva clinical samples using the 11mM GnCl RT-LAMP reaction mix. (A) Table summarizing the results of RT-PCR and RT-LAMP. For both assay, S-gene was not detected for all samples. (B-C) Normalized fluorescence data (n = 3) for the detection of the variant saliva clinical samples with two target-specific primer sets, (B) N-gene and (C) S-gene specific to detect the SGTF. (D) Amplification threshold time was calculated from 20% of fluorescence for both genes. After measuring all samples in the first three replicates, only one amplification occurred in the case of V11 and V15. To confirm this, three more experimental data were added.

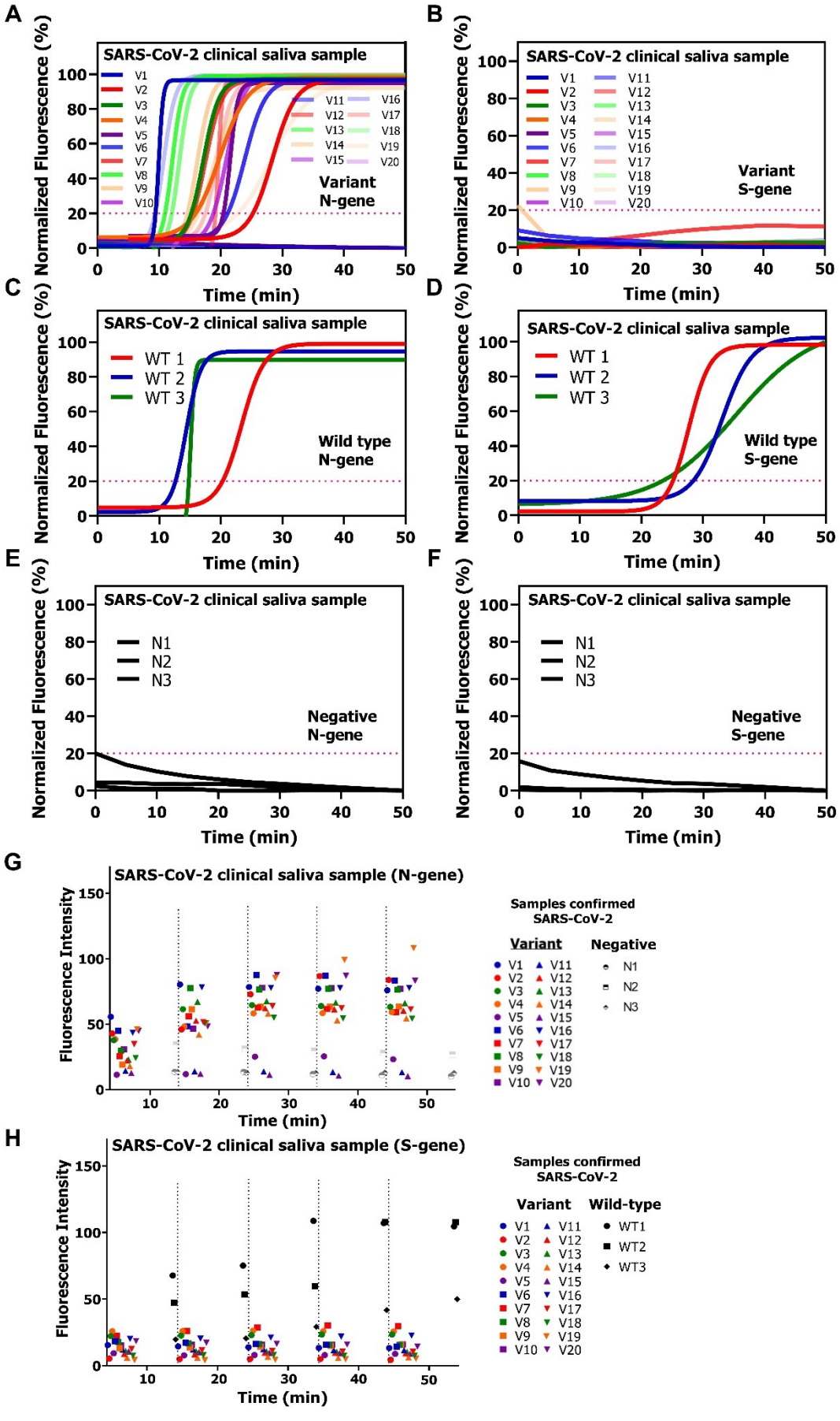

A
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
V1
V2
V3
V4
V5
V6
V7
V8
V9
V10
V11
V12
V13
V14
V15
V16
V17
V18
V19
V20
Variant
N-gene
B
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
Variant
S-gene
C
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
WT 1
WT 2
WT 3
Wild type
N-gene
D
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
WT 1
WT 2
WT 3
Wild type
S-gene
E
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
N1
N2
N3
Negative
N-gene
F
SARS-CoV-2 clinical saliva sample
Normalized Fluorescence (%)
Time (min)
N1
N2
N3
Negative
S-gene
G
SARS-CoV-2 clinical saliva sample (N-gene)
Fluorescence Intensity
Time (min)
Samples confirmed
SARS-CoV-2
Variant
Negative
N1
N2
N3
H
SARS-CoV-2 clinical saliva sample (S-gene)
Fluorescence Intensity
Time (min)
Samples confirmed
SARS-CoV-2
Variant
Wild-type
WT1
WT2
WT3

Figure S11. On-cartridge detection of SARS-CoV-2 B.1.1.7 variant clinical sample and its differentiation from the SARS-CoV-2 early strains and negative samples. (A-B) Normalized fluorescence amplification curves for SARS-CoV-2 detection, (A) N-gene (B.1.1.7 variant saliva samples); (B) S-gene (B.1.1.7 variant saliva samples), (C) N-gene (SARS-CoV-2 early strains saliva samples); (D) S-gene (SARS-CoV-2 early strains saliva samples); and (E) N-gene (negative clinical samples); (F) S-gene (negative clinical samples). (G-H) Raw fluorescence data for (G) N-gene and (H) S-gene specific to SGTF.

Table S1. Binary strain decision table based on the amplification fluorescence results.

Differentiation assay		Strain decision
N-gene	S-gene specific to detect SGTF	
1	1	Early Strains
1	0	B.1.1.7 variant
0	0	Negative

Table S2. Comparison of VTM and saliva samples collected from the same patients. On the same day as saliva collection, nasal swab samples were also collected from the same subject at Carle Foundation Hospital and analyzed by the RT-PCR technique at the Carle clinical lab.

Sample ID	VTM	Saliva
S1	POS	POS
S2	POS	POS
S3	POS	POS
S4	POS	POS
S5	POS	POS
S6	POS	POS
S7	POS	POS
S8	POS	POS
S9	POS	POS
S10	POS	POS
S11	POS	POS
S12	POS	NEG
S13	POS	NEG
S14	POS	NEG
S15	POS	NEG
S16	NEG	NEG
S17	NEG	NEG
S18	NEG	NEG

Table S3. Comparison of various detection methods of SARS-CoV-2.

No.	Technology	LOD (copies/μL)	Sensitivity (%)	Specificity (%)	Assay time (min)	POC	Variant detection	Reference
1	RT-PCR	0.1 - 100	90-100	100	120 - 140	Low	No	1-2
2	RT-LAMP	0.75 - 75	90	100	30 - 60	High	No	2-3
3	CRISPR	5 - 20	86	100	45 - 70	High	No	2-3
4	Our approach	10	91	100	30	High	Yes	N/A

References

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[2] Giri, Basant, Shishir Pandey, Retina Shrestha, Krisha Pokharel, Frances S. Ligler, and Bhanu B. Neupane. "Review of analytical performance of COVID-19 detection methods." Analytical and bioanalytical chemistry 413, no. 1 (2021): 35-48.

[3] Yu, Choo Yee, Kok Gan Chan, Chan Yean Yean, and Geik Yong Ang. "Nucleic acid-based diagnostic tests for the detection SARS-CoV-2: an update." Diagnostics 11, no. 1 (2021): 53.

Results 1. Fluid mixing characterization

To ensure that the fluid is well mixed before entering the detection region, we design the mixer element geometry to provide mixing. We do not know the fluid distribution as it exits the loading region and enters the flow channel of the mixer, so we consider two potential scenarios that represent a worst case unmixed.

In the first scenario, the two fluids enter the region parallel to each other in the channel distributed along the width of the channel. The fluids interdiffuse across the width of the channel as they flow in the axial direction. For this case we calculate the Peclet number (*Pe*) as $Pe = UL/D$ where U is the mean velocity, L is the characteristic length of the channel, and D is the diffusivity. In our flow, U = 3 mm/s, L = 0.51mm, and D = 1E-9 m^2/s which yields Pe = 1530. The mixing flow length required for mixing in a straight channel is $L_{M1} = Pe*L$ = 780 mm [1].

In the second scenario, the fluids enter the mixer sequentially – first the sample and then the premixed reagents. Here we consider Taylor Dispersion in which the fluids interdiffusion in the axial flow direction. We can calculate a second mixing length as $L_{M1} = U*t$ with $t \sim L^2/D$, where U, D, L are the same as the previous scenario and t is the mixing time [2]. For this flow, L_{M2} = 600 mm.

These estimates of mixing length are likely conservative, as there is some mixing that likely occurs in the loading region. Furthermore, the flow through serpentine channels is known to enhance mixing through stretching and folding mechanisms [3, 4, 5]. Thus, we believe that our micromixer with a length of 600 mm and 40 right angle turns provides adequate mixing.

References

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[2] Probstein, R. F. (2003). *Physicochemical hydrodynamics: An introduction*. Wiley.

[3] Ottino, J. M. (2004). *The kinematics of mixing: Stretching, Chaos, and transport*. Cambridge Univ. Press.

[4] Liu, R. H. et. al. (2000). Passive mixing in a three-dimensional serpentine microchannel. *Journal of Microelectromechanical Systems*, *9*(2), 190–197. https://doi.org/10.1109/84.846699.

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Results 2. RT-LAMP assay characterization

The new RT-LAMP assay was first characterized off-cartridge using inactivated SARS-CoV-2 virus spiked in healthy human saliva (Lee Biosolutions). As part of this characterization, the sensitivity of the assay and the limit of detection (LOD) were calculated. Gamma-Irradiated SARS-CoV-2 virus (NR-52287, BEI) aliquots were diluted to the desired concentrations by spiking them into the saliva sample, followed by thermal lysis (95°C, 10 min.). Lysed samples were mixed with TE buffer (5% Tween 20). Then, 2 µL of samples were loaded together with 14 µL of RT-LAMP reaction master mix (1:7 ratio) into 0.2 mL PCR tubes. The amplification reaction was performed in the QuantStudio 3 system (65°C, 50 min.) and fluorescence changes were measured every minute. EvaGreen dye, a double strand intercalating dye, was used as a fluorophore to quantify the amount of resulting amplicons during the amplification. Samples with inactivated SARS-CoV-2 virus, in the range of 10^4 to 0 copies/µL, spiked in human saliva were tested. Normalized fluorescence values and amplification threshold times for both N- and S-genes can be found in (supplementary information, **Figs. S1C-F**). The amplification threshold (denoted with a pink dotted line) was calculated as 20% of the maximum fluorescence signal. The negative control (0 copy/µL) did not show amplification in any of the replicates. The LOD of the RT-LAMP assay was found to be ≤ 10 copies/µL for both N- and S-gene target specific assay.

Results 3. Single target on-cartridge detection

Since the on-cartridge and cradle setups entail a variety of possible variabilities to the reaction conditions, we first examined the feasibility of the on-cartridge N-gene virus detection using the AM cartridge with single detection reservoir (supplementary information, **Fig. S4**). Protocols for the spiked sample treatment and reagent preparation were the same as those from the off-cartridge experiments. However, instead of mixing them into 0.2 mL PCR tubes, the sample and reaction mix were loaded into the inlet region of the AM cartridge (6 + 42 µL, 1:7 ratio). Both solutions were driven through the 3D mixing serpentine by pulling the syringe connected to the outlet port. Once the detection reservoir was filled, the inlet and outlet ports of the cartridge were sealed, and the cartridge was placed on an external hot plate (set to maintain a fluid temperature of 65°C) to start the amplification event. Thereafter, the LEDs and the blue filters, located on the cartridge, are used for the excitation of the dye and to pass only the emitted fluorescence. The changes in fluorescence inside the detection reservoir were monitored for 50 minutes in real-time using a smartphone (images were taken every 5 min.). **Fig. S4A** (supplementary information) shows a schematic of the cartridge used for this characterization. As an example, fluorescence images taken with the smartphone for the real-time detection of 10^3 and 10^2 copies/µL of SARS-CoV-2 virus spiked in saliva are shown in **Fig. S4B** (supplementary information). Gradual increase of the fluorescence indicates the formation of amplicons and more of the EvaGreen dye conjugated into the double-strand DNA. The collected images were analyzed for the quantification of the fluorescence intensity using the ImageJ software. For this analysis, four different locations of the illuminated area were selected to represent the entire detection reservoir. The intensity of these four locations was averaged, and these values were used to build the amplification curves for SARS-CoV-2 virus concentration in the range from 10^4 to 0 copies/µL. From the normalized data (supplementary information, **Fig. S4C**), the amplification threshold times were calculated at 20% of the maximum intensity (denoted with a pink dotted line in **Fig. S4C** and plotted in **Fig. S4D**, supplementary information). Negative controls did not show amplification. Thus, the LOD was found to be ≤ 10 copies/µL, which is consistent with the off-cartridge results. Finally, a calibration curve for the detection of a single target on the cartridge was built and is shown in **Fig. S4E** (supplementary information).

Results 4. Assay validation for detection of SARS-CoV-2 (early strains) from clinical saliva samples

We performed the multiplexed off-cartridge RT-LAMP-based detection of SARS-CoV-2 virus (early strains) from clinical saliva samples on the AM cartridge. Eighteen (18) clinical saliva samples were obtained from the Carle Foundation Hospital through an approved IRB. Received samples were aliquoted and stored at -80°C until further use. All 18 clinical saliva samples were tested by both off- and on-cartridge experiments. The RT-LAMP results were compared with the RT-qPCR controls. The RT-qPCR protocol was as follows: Preparation of the samples has gone through the same procedures as explained before for the inactivated virus samples. Briefly, aliquots were thermally lysed (95°C, 30 minutes), followed by mixing with 2x TBE with 1% Tween-20 buffer (1:1 ratio). A 5 µL sample mixture was used for a real-time RT-PCR with a total of 10-µL volume using TaqPath 1-step Multiplex Master Mix (no ROX) and TaqPath™ COVID-19 Combo Kit (ThermoFisher Scientific). Each reaction of COVID-19 PCR mastermix includes 2.5 µL TaqPath 1-step Multiplex Master Mix (no ROX), 0.5 µL COVID-19 real-time PCR assay multiplex, 1.025 µL MS2 phage control and 1.15 µL DNase and RNase free water. The real-time RT-PCR was ran on a QuantStudio 7 Pro machine with thermocycler conditions 25°C 2 min., 53°C 10 min., 95°C 2 min., and 40 cycles of 95°C 3 sec. and 60°C 30 sec. PCR data were analyzed on Design and Analysis Software 2.4.3 version ThermoFisher Scientific, and sample were called positive or negative using the interpretive scheme of the kit manual. First, the saliva samples were examined by a RT-PCR assay, the current gold standard, to establish the control data for the comparison with our RT-LAMP assay. Three target genes were analyzed using three different reporters, VIC, FAM and ABY for the N-, ORF1ab- and S-genes respectively. The RT-PCR Ct values are shown in **Fig. S6A** (supplementary information). From the RT-PCR control assay, eleven samples (S1-S11) were identified as positives, while 7 samples (S12-S18) were identified as negatives. Next, off-cartridge RT-LAMP was conducted with the 2 µL of sample, followed by mixing it with 14 µL of RT-LAMP reaction master mix in the 0.2 mL PCR tubes. Two experiments of RT-LAMP specific to N- and S-gene were performed (65°C, 50 minutes), separately. The normalized amplification curves and amplification threshold times of the 18 clinical samples tested by RT-LAMP assay are shown in **Figs. S6B-E** (supplementary information). As can be seen in **Fig. S6A**, the saliva samples confirmed negative by the RT-PCR control (S12-S18), were also confirmed negative by the RT-LAMP assay (none of the genes showed amplification). As a result, our off-cartridge assay achieves 91% sensitivity and 100% specificity when analyzing SARS-CoV-2 early strains clinical saliva samples.