Supplementary Information

3’ end hydrolysis activity of the Bst polymerase enables rapid and sensitive identification of SARS-CoV-2 variants

Kean Hean Ooi[1,4], Mengying Mandy Liu[1,4], Muhammad Safwan Bin Burhanudin[1], Surenthar Thirunavukarasu[1], Zhi Qi Victor Oh[1], Shiuan Ning Shannon Kwa[1], Tian Hui Valerie Ng[1], Pattaraporn Nimsamer[2], Sunchai Payungporn[2], Pornchai Kaewsapsak[2], Meng How Tan[1,3,*]

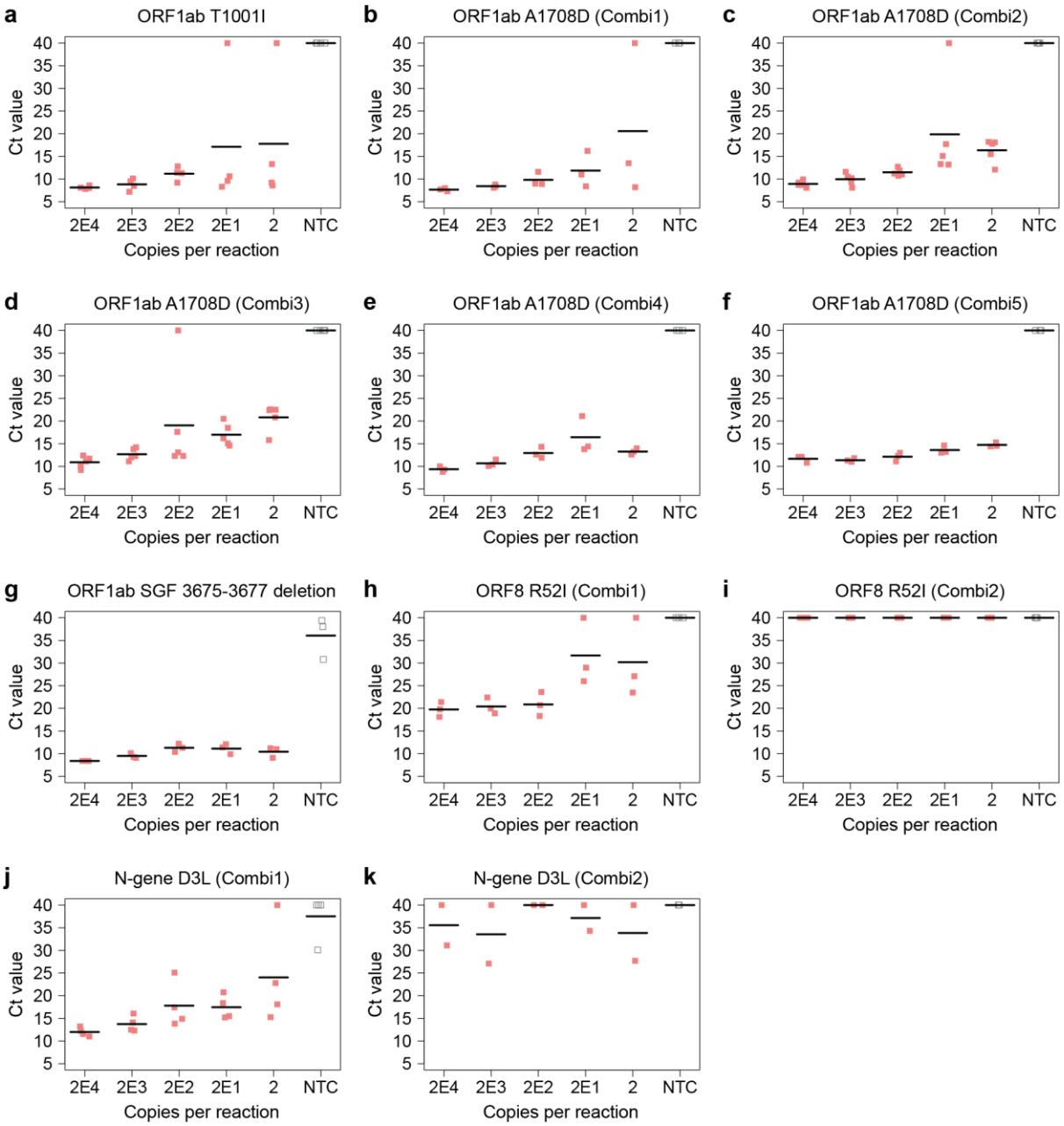
[1]School of Chemistry, Chemical Engineering and Biotechnology, Nanyang Technological University, Singapore 637459, Singapore

[2]Center of Excellence in Systems Microbiology, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

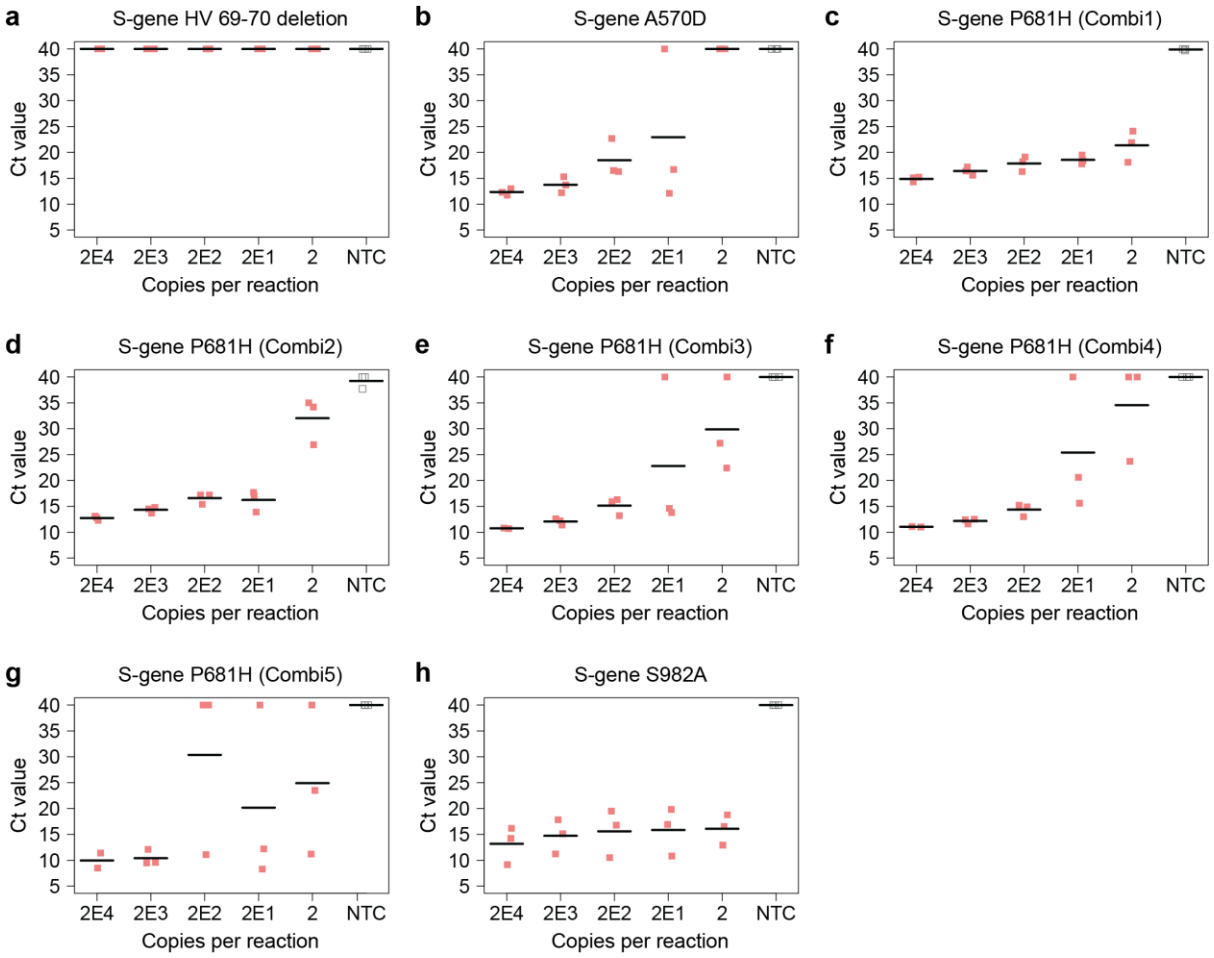
[3]HP-NTU Digital Manufacturing Corporate Lab, Nanyang Technological University, Singapore 637460, Singapore

[4]These authors contributed equally.

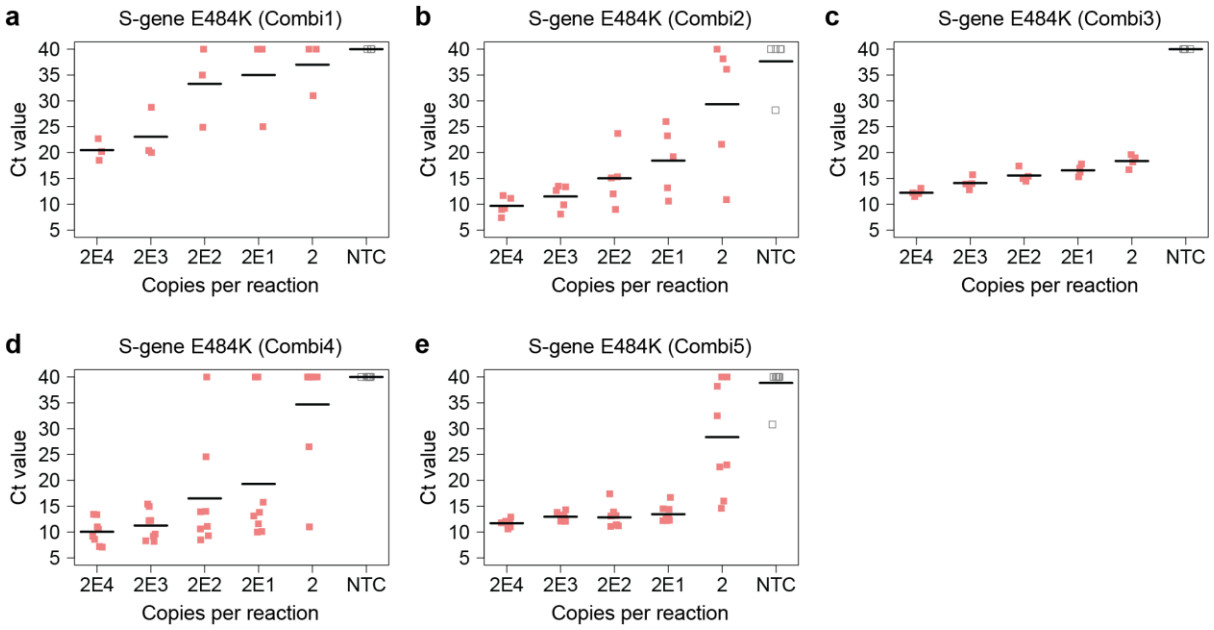
[*]Correspondence: mh.tan@ntu.edu.sg



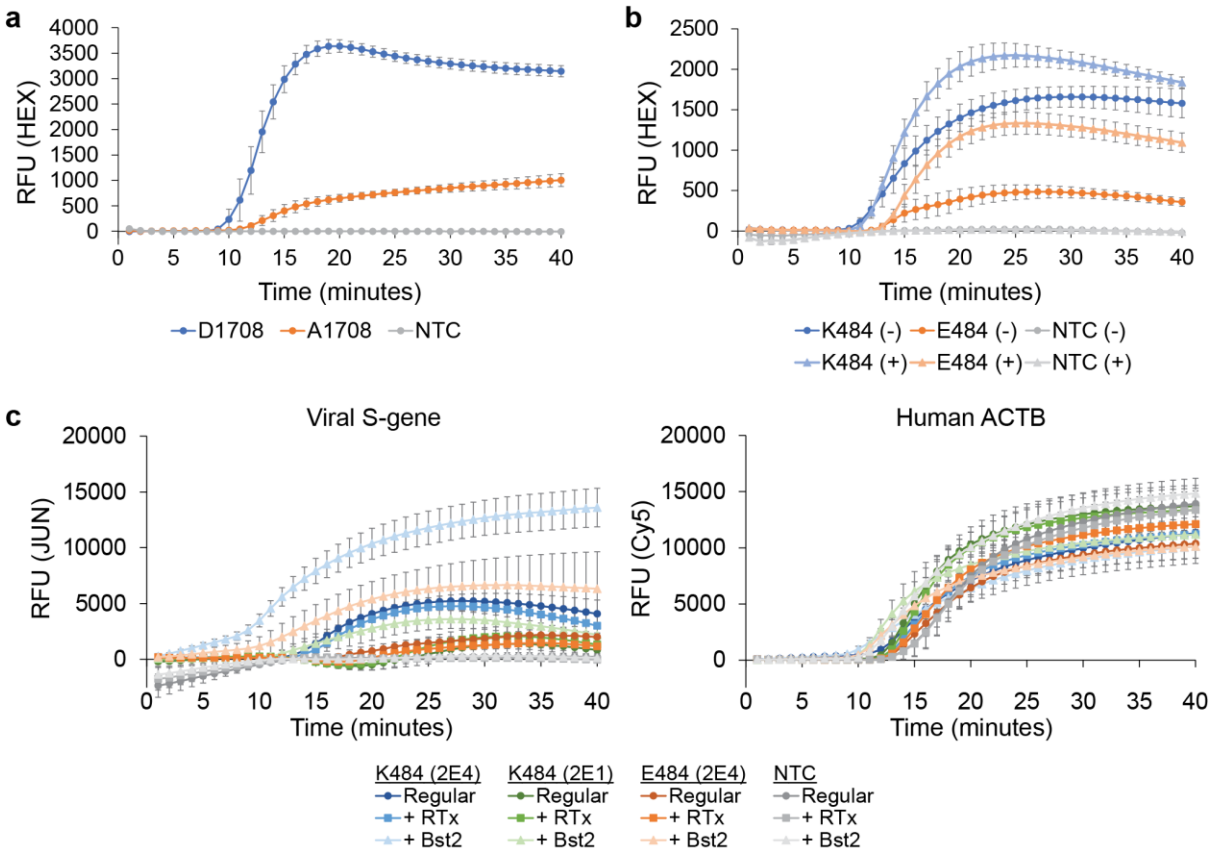
Supplementary Figure 1. Evaluation of LAMP primers for non-S-gene mutations found in the Alpha variant. The black horizontal bars among the data points in the strip charts represent the mean (n = 2 [**k**], 3 [**b**, **e-i**], 4 [**a**, **j**], or 5 [**c-d**] biological replicates).



Supplementary Figure 2. Evaluation of LAMP primers for S-gene mutations found in the Alpha variant. The black horizontal bars among the data points in the strip charts represent the mean (n = 2 [P681H (Combi3-5) 2E4 copies] or 3 [all others] biological replicates).



Supplementary Figure 3. Screening of multiple sets of LAMP primers for the E484K mutation in the S-gene of SARS-CoV-2. The black horizontal bars among the data points in the strip charts represent the mean (n = 3 [**a**], 4 [**c**], 5 [**b**], or 8 [**d-e**] biological replicates).

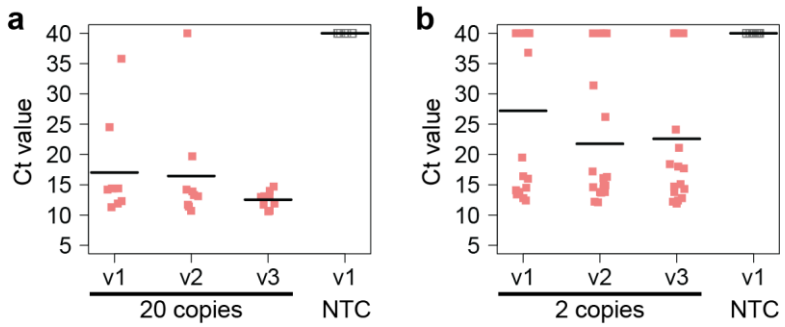


Supplementary Figure 4. Time courses of the fluorescence intensity in our RT-LAMP assays containing different LANTERN probes.

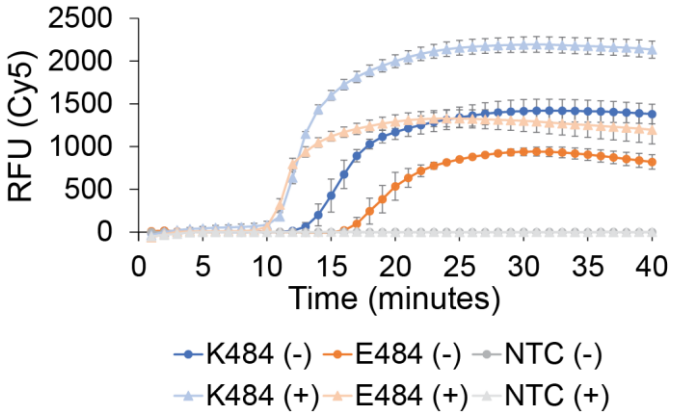
a. Evaluation of a probe targeting the A1708D mutation in the ORF1ab gene of SARS-CoV-2. 2E4 copies of in vitro transcribed RNA templates corresponding to A1708 or D1708 were used as template. Data represent mean ± s.e.m. (n = 4 biological replicates).

b. Evaluation of a probe targeting the E484K mutation in the S-gene of SARS-CoV-2. 2E4 copies of in vitro transcribed RNA templates corresponding to E484 or K484 were used as template. 0.5U of Q5 High-Fidelity DNA Polymerase was also added in each reaction mix. Here, RT-LAMP reactions were performed without (-) or with (+) swarm primers. Data represent mean ± s.e.m. (n = 5 biological replicates).

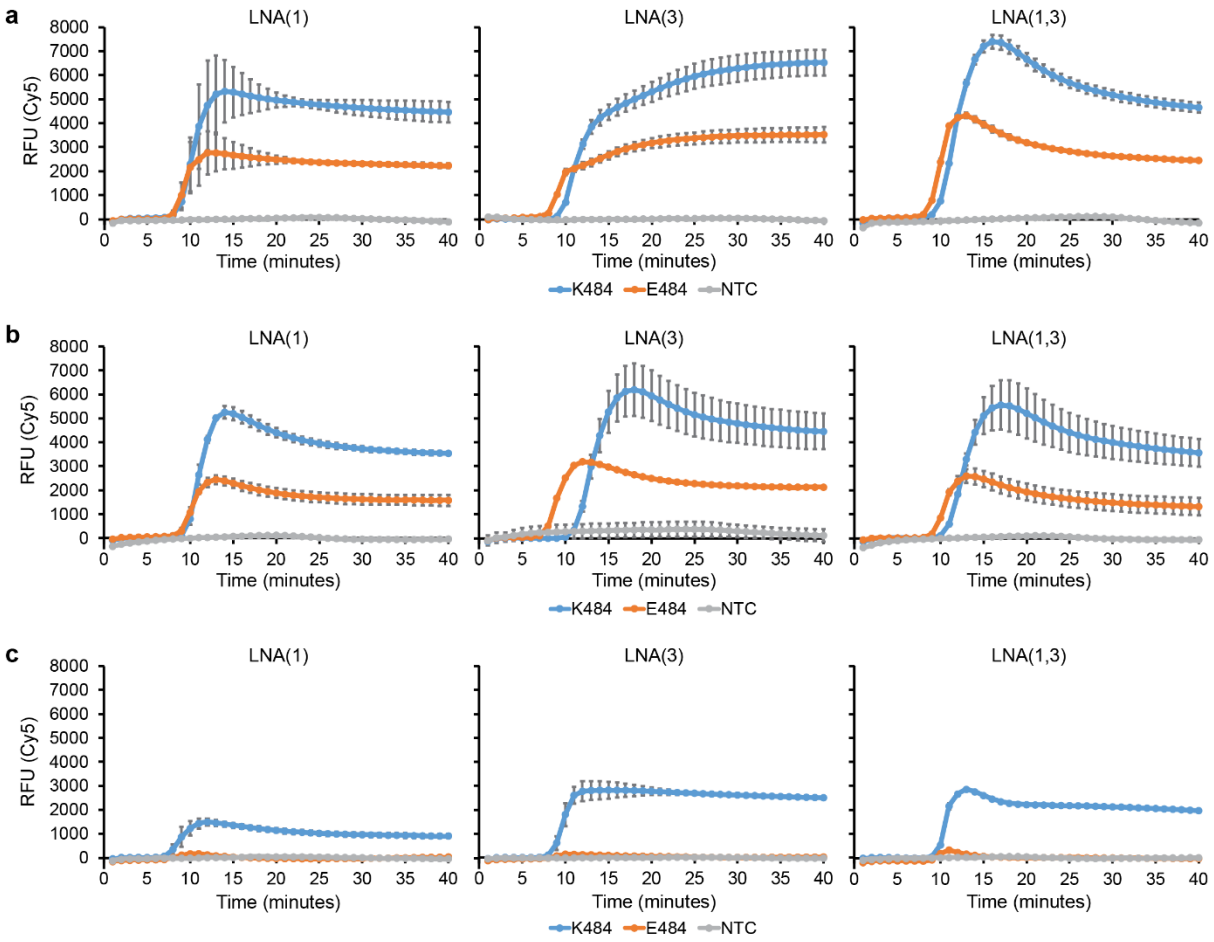
c. Assessing the effect of additional reverse transcriptase or Bst2 enzyme. In vitro transcribed S-gene templates (corresponding to E484 or K484) were spiked into 0.25ng of PC9 RNA. 0.5µM K484-targeting LANTERN probe was used with a S-gene LAMP primer set that excluded the swarm primers. 0.5U of Q5 High-Fidelity DNA Polymerase was also added in each reaction. Fluorescence in the Cy5 channel (for human ACTB) and the Texas-Red channel (for viral S-gene) was monitored over the course of the reactions. Data represent mean ± s.e.m. (n = 4 biological replicates).



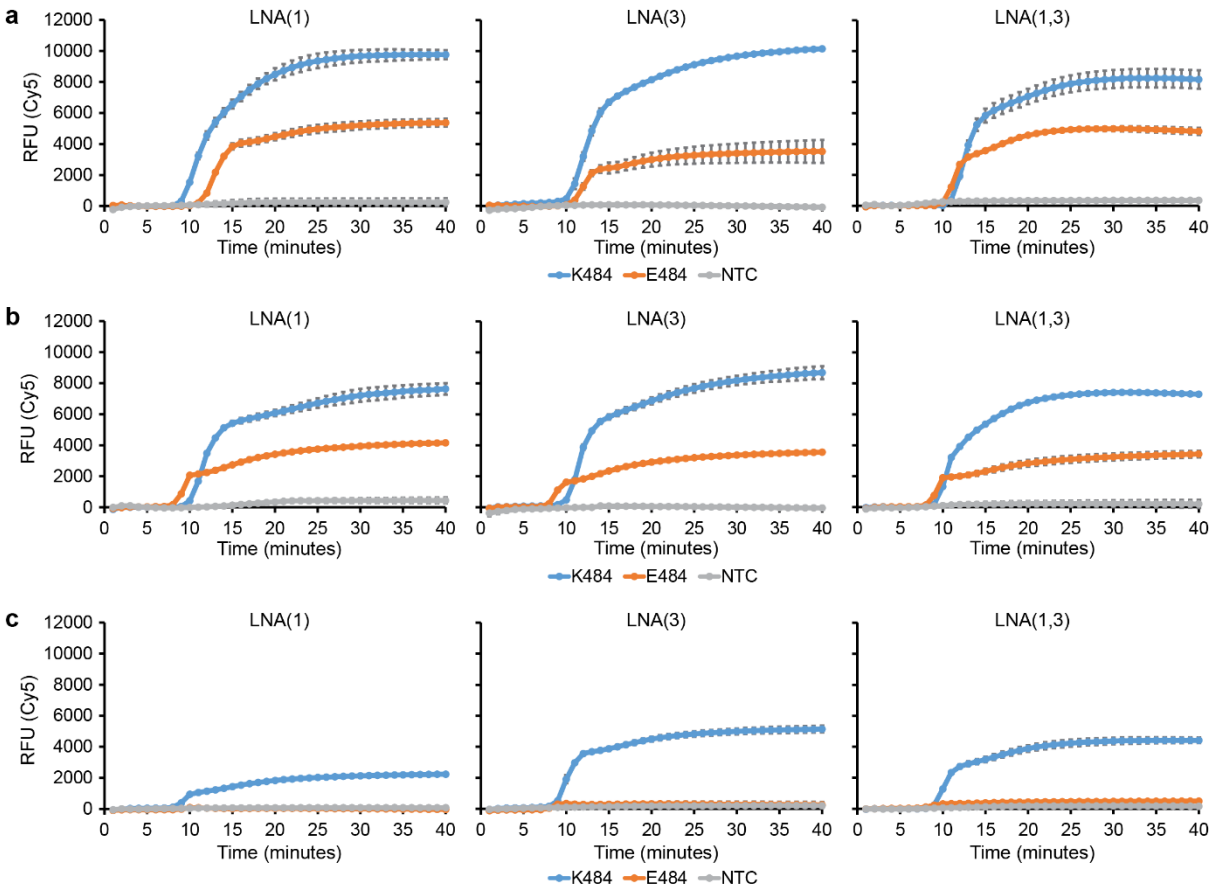
Supplementary Figure 5. Optimization of displacement primers, which are known to be important for the kinetics of LAMP reactions. Three different B3 primers were evaluated using either **a** 20 copies or **b** 2 copies of synthetic S-gene (K484) RNA template per reaction. The black horizontal bars among the data points in the strip charts represent the mean (n = 9 [**a**] or 18 [**b**] biological replicates).



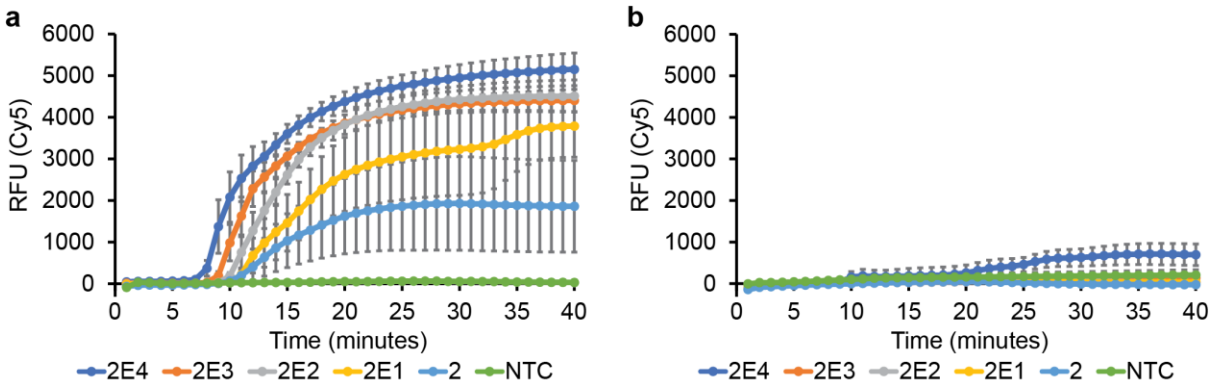
Supplementary Figure 6. Evaluating the effect of extra Bst2 enzyme on the performance of our new probe method. Here, 0.5µM of 22nt LNAnt1 probe targeting the K484 template was used without swarm primers. 2E4 copies of in vitro transcribed RNA (either K484 or E484) were added in each sample as template. The RT-LAMP reactions were carried out without (-) or with (+) extra 8U Bst2 DNA polymerase. Data represent mean ± s.e.m. (n = 3 biological replicates).



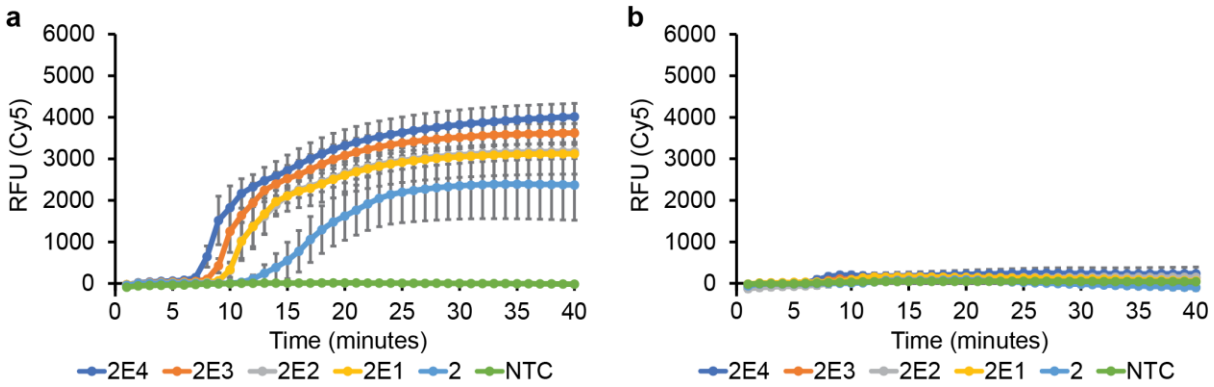
Supplementary Figure 7. Time courses of the fluorescence intensity in our RT-LAMP assays containing K484-targeting SNiPER probes of lengths **a** 23nt, **b** 22nt, and **c** 21nt. No swarm primers were used. Data represent mean ± s.e.m. (n = 2 [23nt LNA(3) and LNA(1,3); 22nt; 21nt LNA(1,3)], 3 [23nt LNA(1)], or 4 [21nt LNA(1) and LNA(3)] biological replicates).



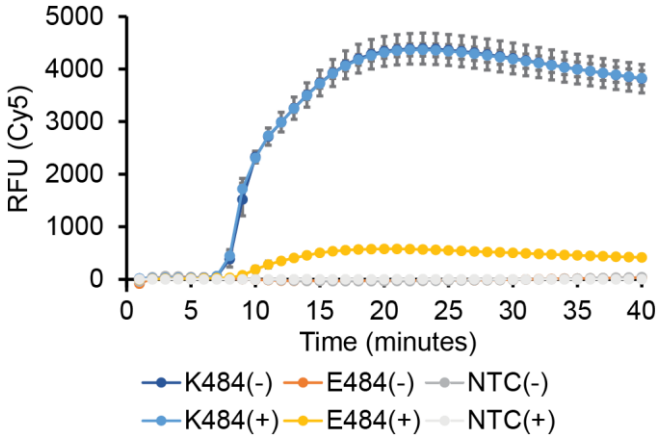
Supplementary Figure 8. Time courses of the fluorescence intensity in our RT-LAMP assays containing K484-targeting SNiPER probes of lengths **a** 23nt, **b** 22nt, and **c** 21nt. Swarm primers were added in the reactions. Data represent mean ± s.e.m. (n = 2 [23nt; 22nt; 21nt LNA(1)] or 4 [21nt LNA(3) and LNA(1,3)] biological replicates).



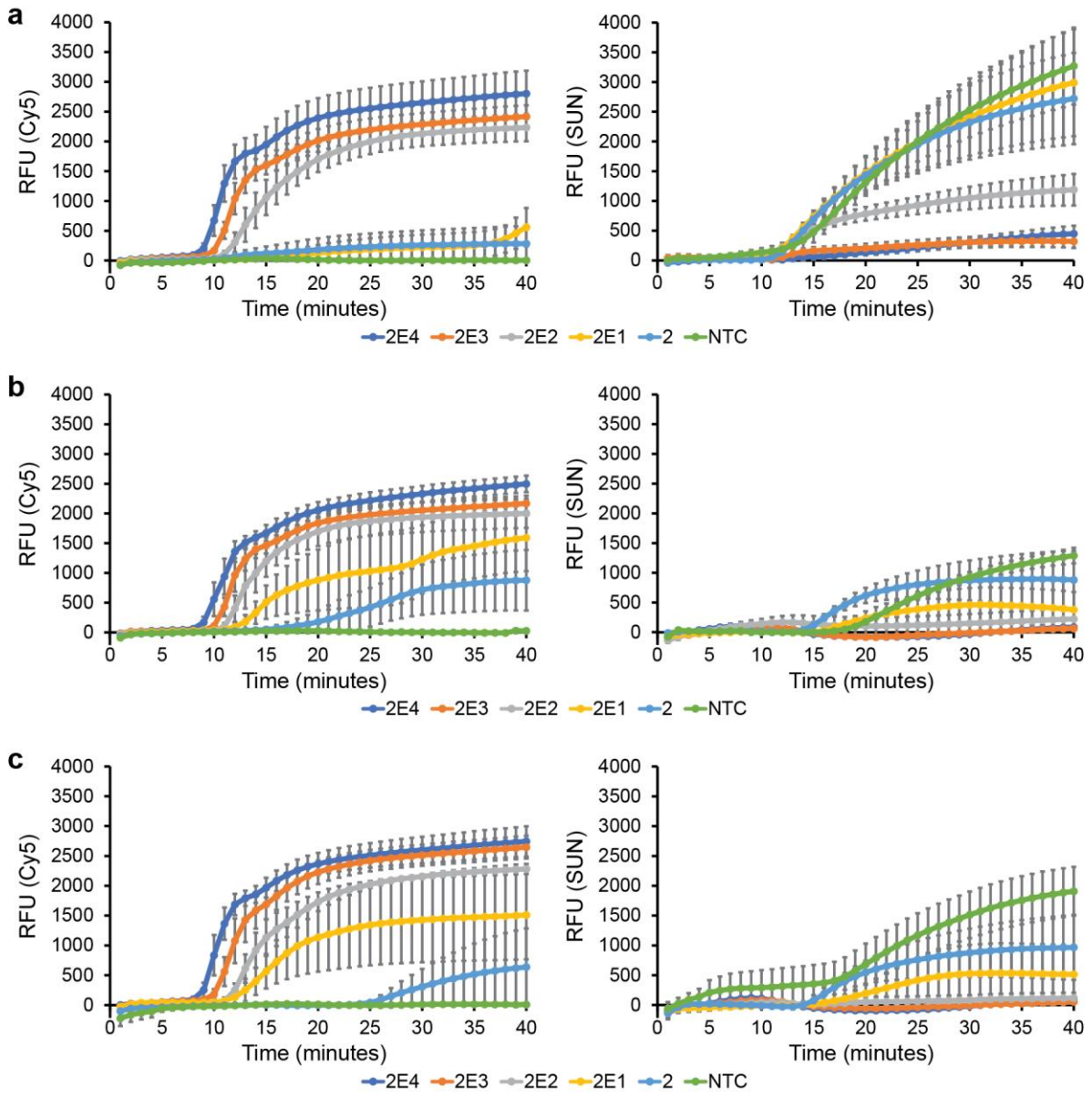
Supplementary Figure 9. Time courses of the fluorescence intensity in our RT-LAMP assays containing synthetic RNA templates encoding either the **a** K484 or **b** E484 version of the viral S-gene. The SNiPER probe was designed to recognize the mutant (K484) template. Data represent mean ± s.e.m. (n = 4 biological replicates).



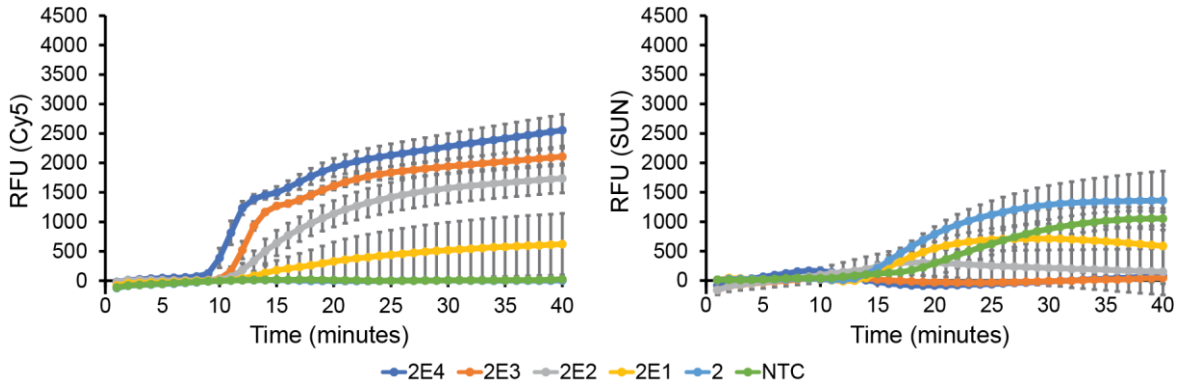
Supplementary Figure 10. Time courses of the fluorescence intensity in our RT-LAMP assays containing lentiviruses packaged with either the **a** K484 or **b** E484 S-gene fragment. Lentiviruses were diluted in TE buffer with 0.1U/µl of Proteinase K and then heated at 95ºC for 5 minutes prior to addition in the reactions. The SNiPER probe was designed to recognize the mutant (K484) template. Data represent mean ± s.e.m. (n = 4 biological replicates).



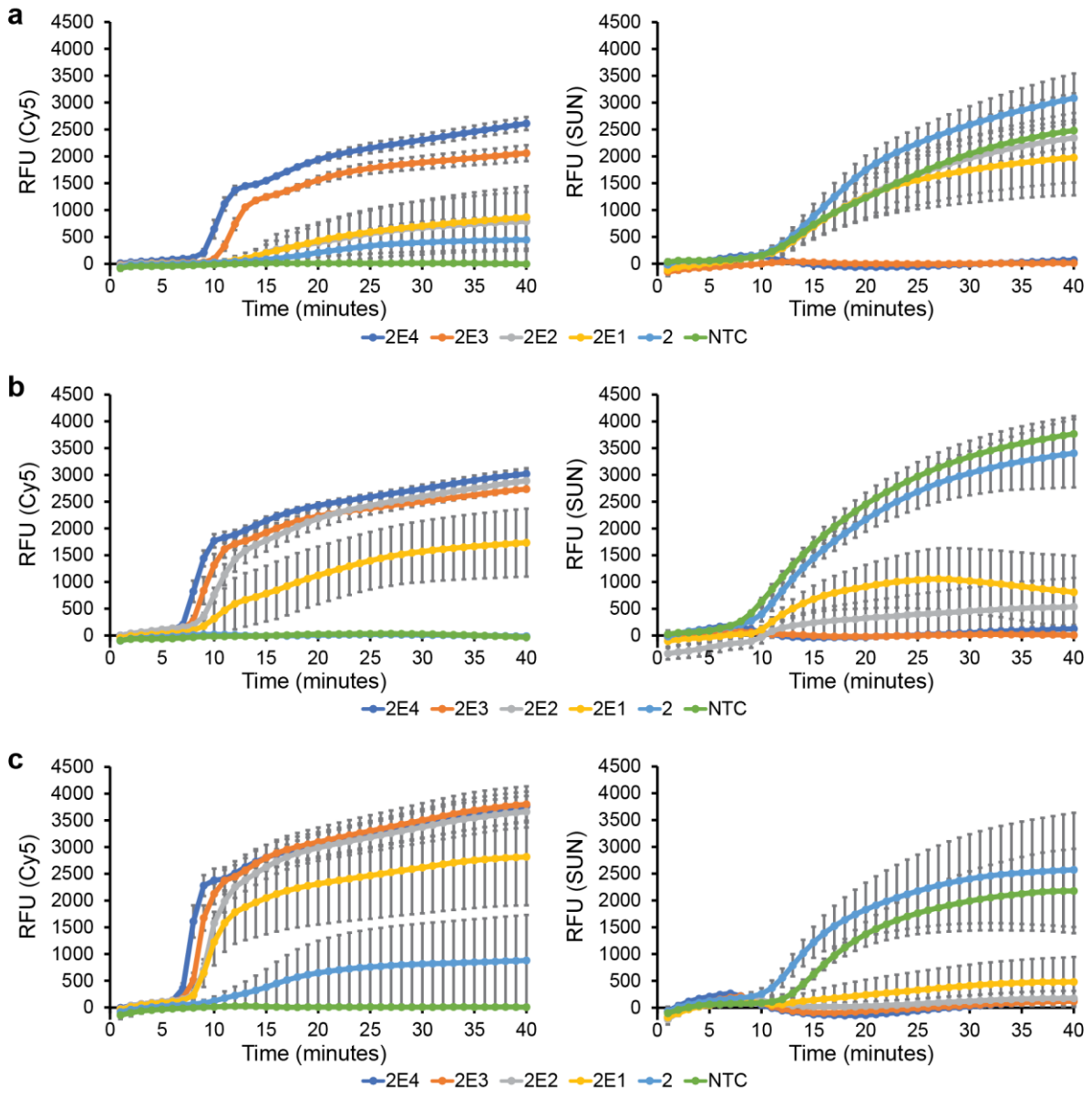
Supplementary Figure 11. Effect of Q5 high-fidelity DNA polymerase on a 21nt LNA(3) SNiPER probe recognizing the K484 mutant viral template. Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



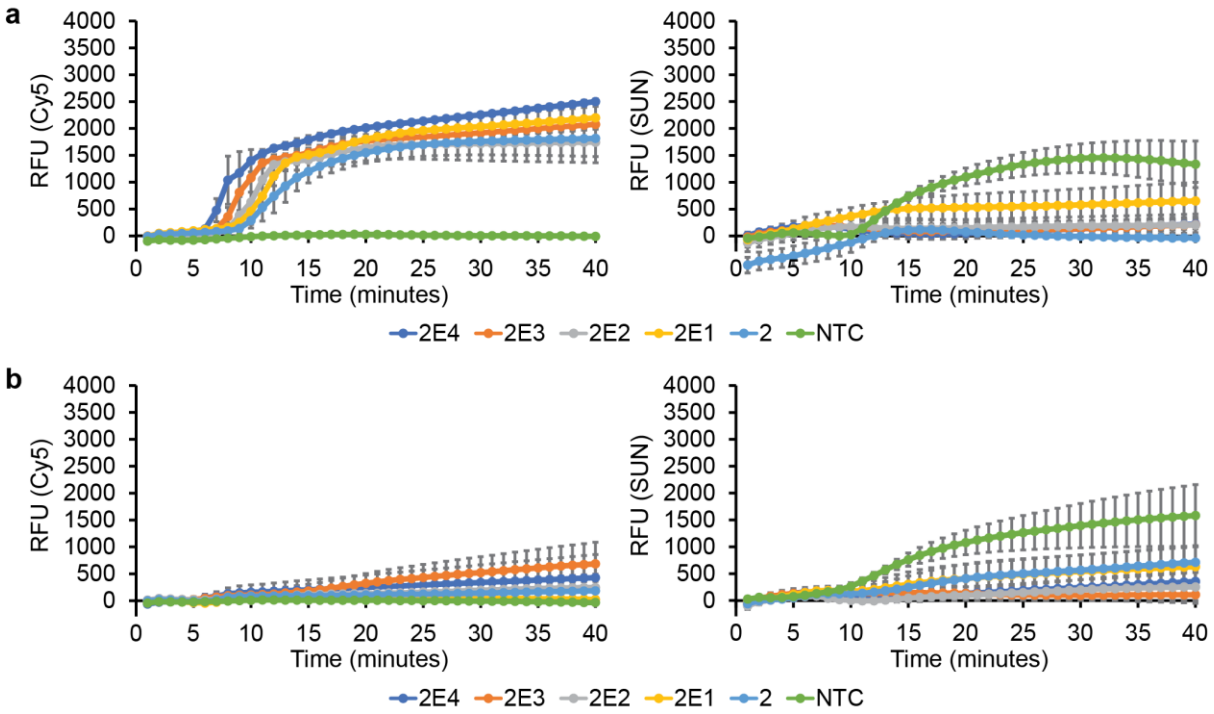
Supplementary Figure 12. Time courses of the fluorescence intensity in 2-plex RT-LAMP assays with human internal control. Different ACTB primer and probe conditions were evaluated: **a** with swarm primers and 0.5µM LANTERN probe, **b** without swarm primers and 0.5µM LANTERN probe, and **c** without swarm primers and 1µM LANTERN probe. Variable copies of synthetic viral RNA with K484 mutation and 0.25ng of PC9 RNA were supplied as template. 0.5U of Q5 DNA Polymerase was also added for detection of the ACTB amplicon. Fluorescence was monitored in the Cy5 channel (S-gene) and the HEX/SUN channel (ACTB). Data represent mean ± s.e.m. (n = 3 [1µM] or 4 [0.5µM] biological replicates).



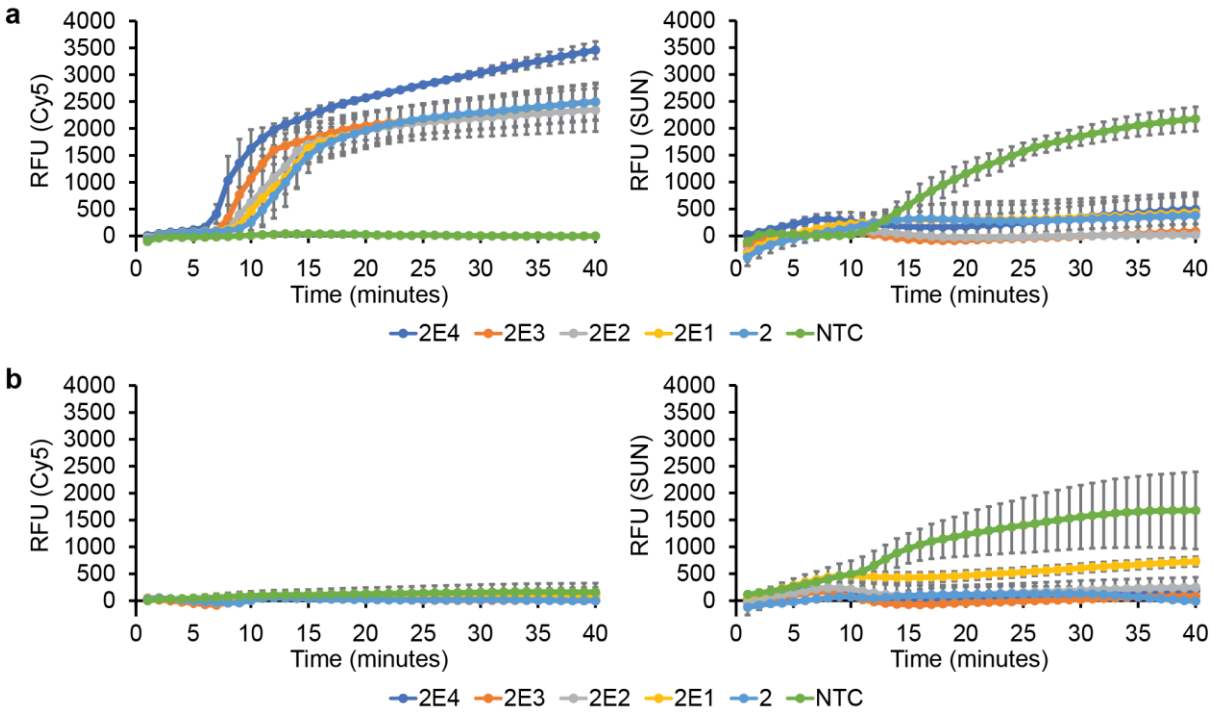
Supplementary Figure 13. Effect of additional reverse transcriptase on 2-plex RT-LAMP reactions with human internal control. 1µM of LANTERN ACTB probe was used without swarm primers. Data represent mean ± s.e.m. (n = 4 biological replicates).



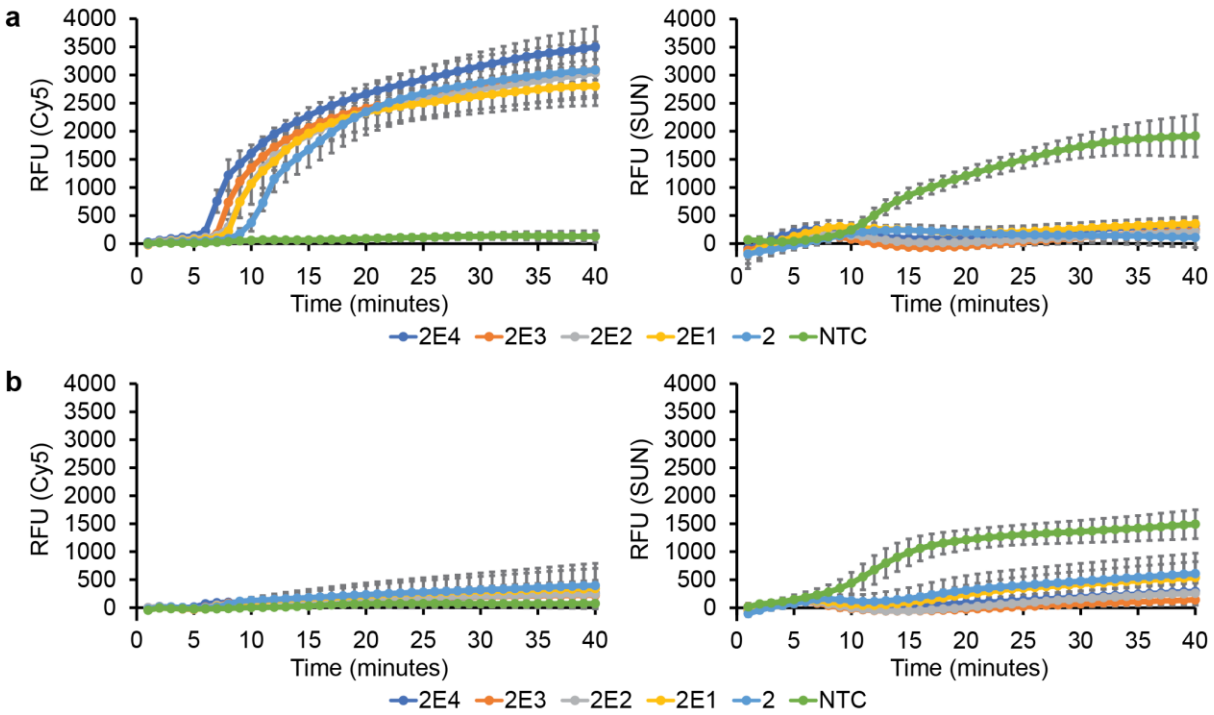
Supplementary Figure 14. Effect of supplementing different Bst DNA polymerases on 2-plex RT-LAMP reactions with human internal control. Bst3.0, Turbo Bst2.0, and IsoPol+ (8U each) were tested. Variable copies of synthetic RNA with K484 mutation and 0.25ng of PC9 RNA were added in each reaction. 1µM of LANTERN ACTB probe was used without swarm primers. 0.5U of Q5 DNA Polymerase was also added for detection of the ACTB amplicon. Fluorescence was monitored in the Cy5 channel (S-gene) and the HEX/SUN channel (ACTB). Data represent mean ± s.e.m. (n = 4 [Bst3.0 and Turbo Bst2.0] or 5 [IsoPol+] biological replicates).



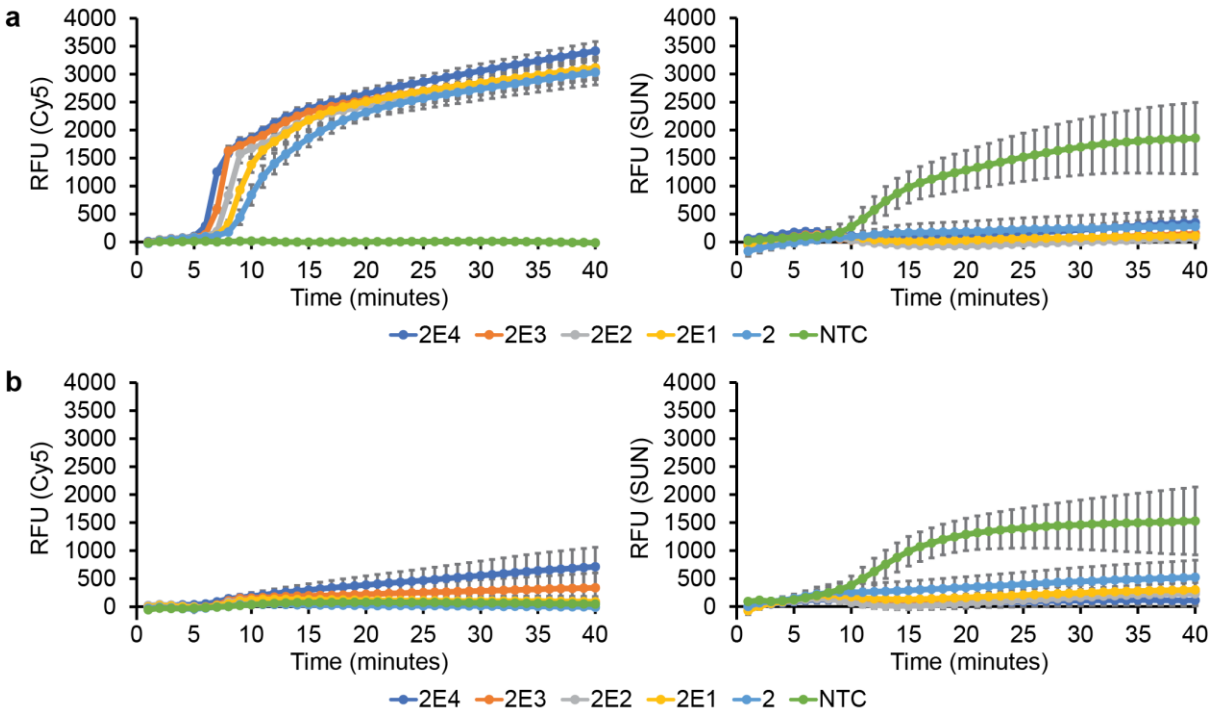
Supplementary Figure 15. Evaluation of saliva samples spiked with variable amounts of lentivirus packaged with either the **a** K484 or **b** E484 S-gene fragment, with each RT-LAMP reaction supplemented with 8U of Turbo Bst2. The SNiPER probe contained a single LNA residue at the -3 position. All the contrived specimens were treated with Proteinase K and 95ºC heat. Data represent mean ± s.e.m. (n = 3 [K484] or 4 [E484] biological replicates).



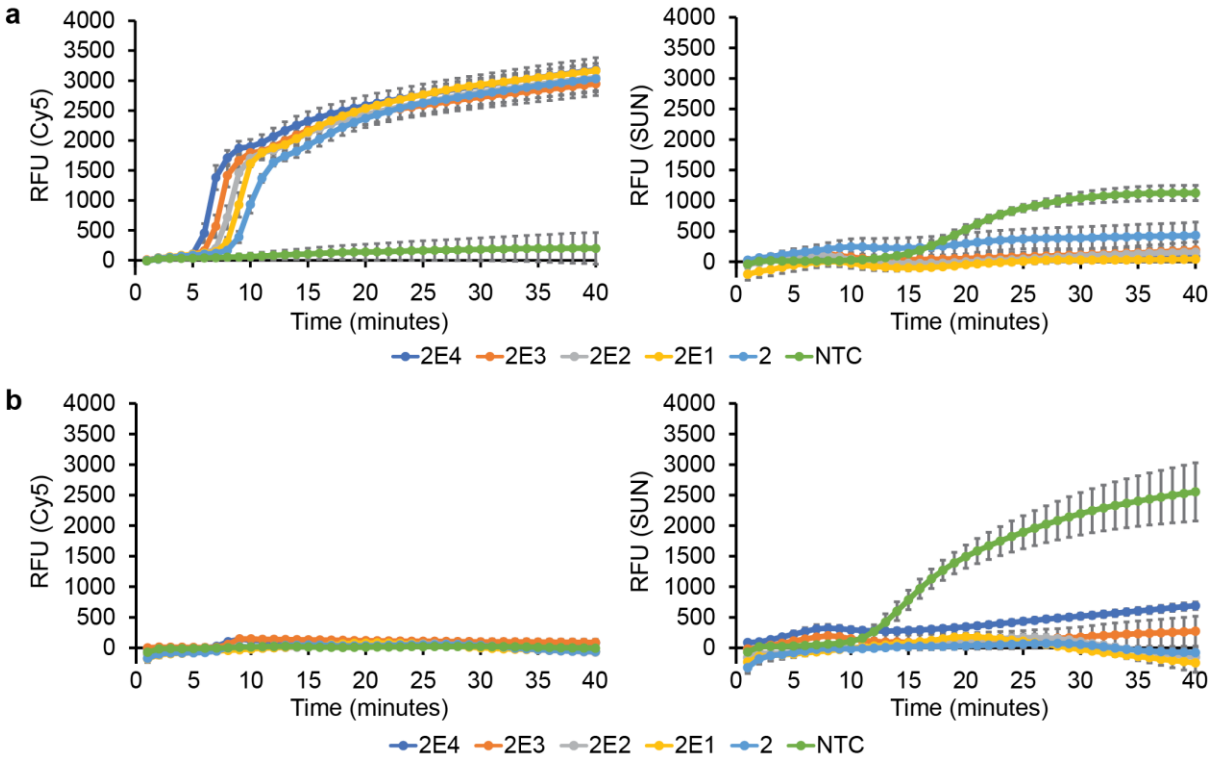
Supplementary Figure 16. Evaluation of saliva samples spiked with variable amounts of lentivirus packaged with either the **a** K484 or **b** E484 S-gene fragment, with each RT-LAMP reaction supplemented with 8U of IsoPol+. The SNiPER probe contained a single LNA residue at the -3 position. All the contrived specimens were treated with Proteinase K and 95ºC heat. Data represent mean ± s.e.m. (n = 3 biological replicates).



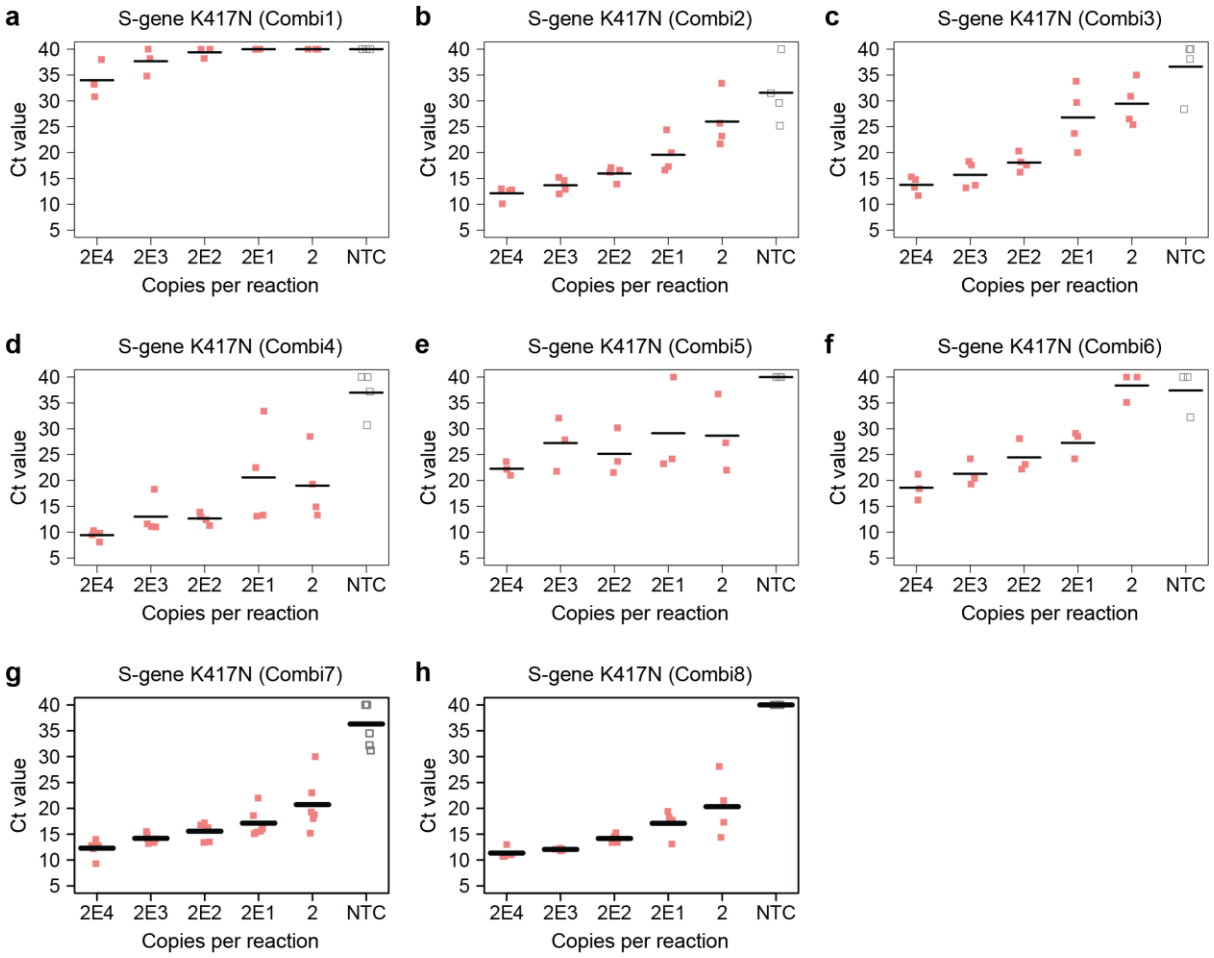
Supplementary Figure 17. Evaluation of saliva samples spiked with variable amounts of lentivirus packaged with either the **a** K484 or **b** E484 S-gene fragment, with each RT-LAMP reaction supplemented with 8U of IsoPol+. The SNiPER probe contained a single LNA residue at the -3 position. All the contrived specimens were treated with Proteinase K, EDTA, and 95°C heat. Data represent mean ± s.e.m. (n = 4 biological replicates).



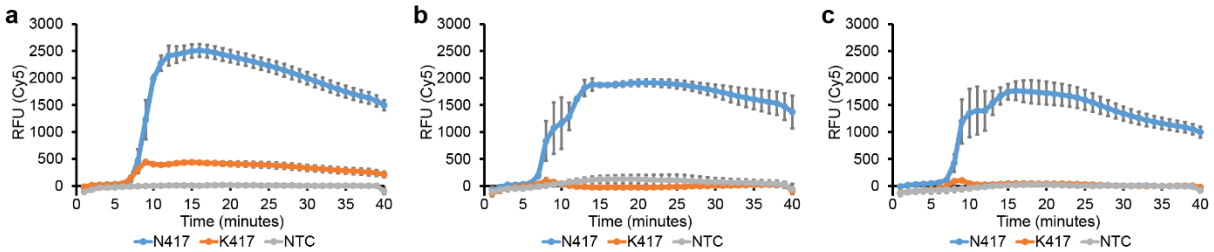
Supplementary Figure 18. Evaluation of saliva samples spiked with variable amounts of lentivirus packaged with either the **a** K484 or **b** E484 S-gene fragment, with each RT-LAMP reaction supplemented with 8U of IsoPol+. The SNiPER probe contained a single LNA residue at the -3 position. All the contrived specimens were processed using a commercially available ZeroPrep Saliva Collection Kit. Data represent mean ± s.e.m. (n = 4 biological replicates).



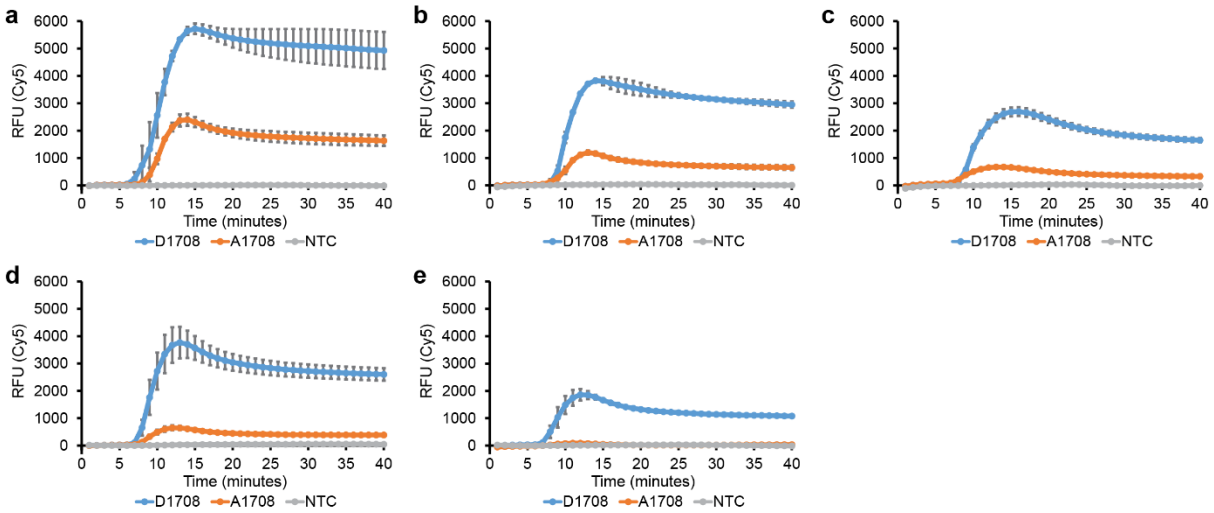
Supplementary Figure 19. Evaluation of saliva samples spiked with variable amounts of lentivirus packaged with either the **a** K484 or **b** E484 S-gene fragment, with each RT-LAMP reaction supplemented with 8U of IsoPol+. The SNiPER probe contained two LNA residues at the -1 and -3 positions. All the contrived specimens were processed using a commercially available ZeroPrep Saliva Collection Kit. Data represent mean ± s.e.m. (n = 3 [K484; E484 2/2E1/2E2/2E3/2E4] or 5 [E484 NTC] biological replicates).



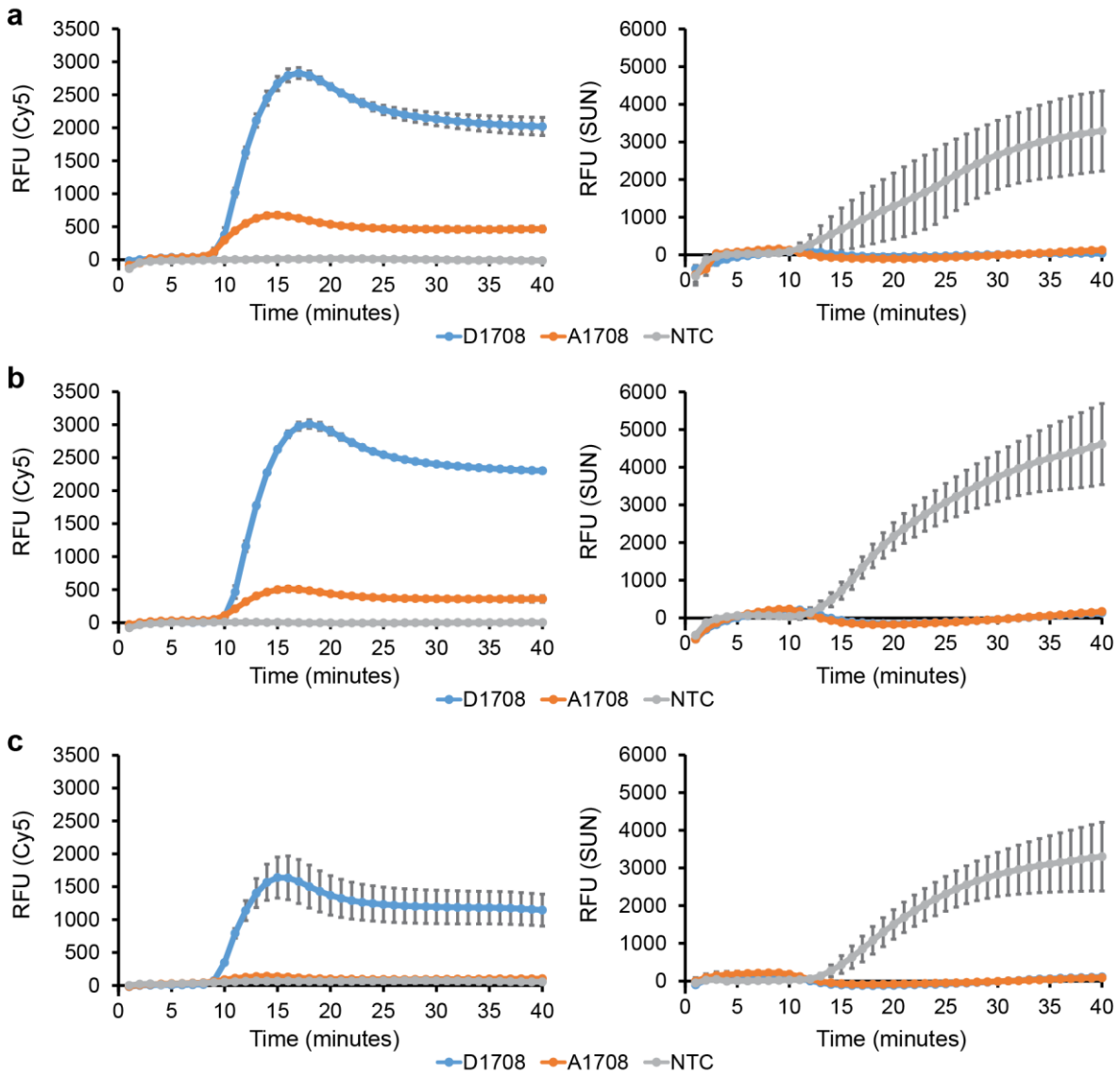
Supplementary Figure 20. Screening of multiple sets of LAMP primers for the K417N mutation in the S-gene of SARS-CoV-2. Data represent mean ± s.e.m. (n = 3 [Combi1, Combi5, Combi6], 4 [Combi2, Combi3, Combi4, Combi8], or 6 [Combi7] biological replicates).



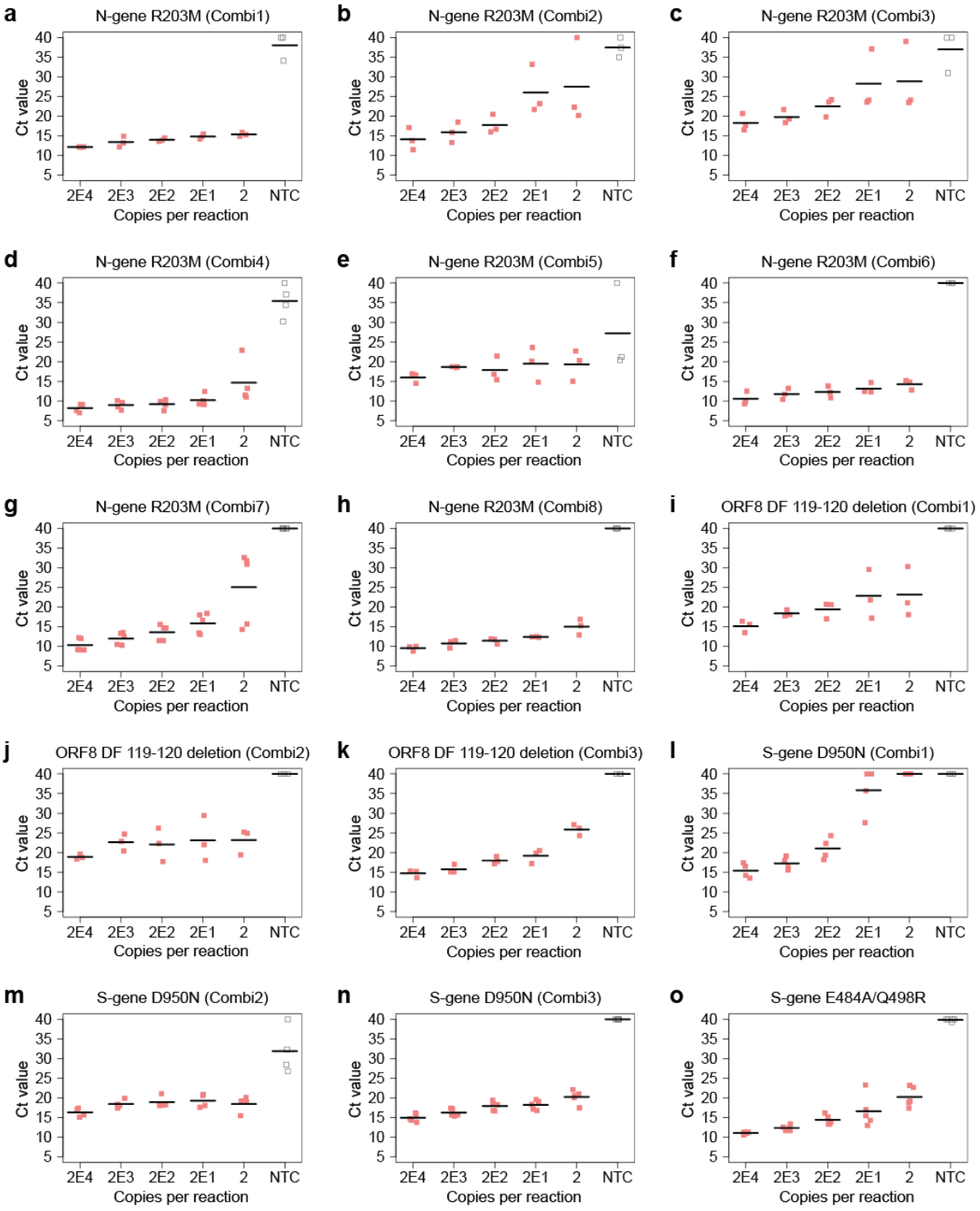
Supplementary Figure 21. Evaluation of various SNiPER probes for detection of K417N in the S-gene. 2E4 copies of wildtype (K417) or mutant (N417) synthetic RNA fragments were used as template. Three probes were examined at 0.5µM concentration, namely **a** 19nt−LNA(3), **b** 18nt−LNA(3), and **c** 17nt−LNA(3). Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 [18nt] or 4 [17nt, 19nt] biological replicates).



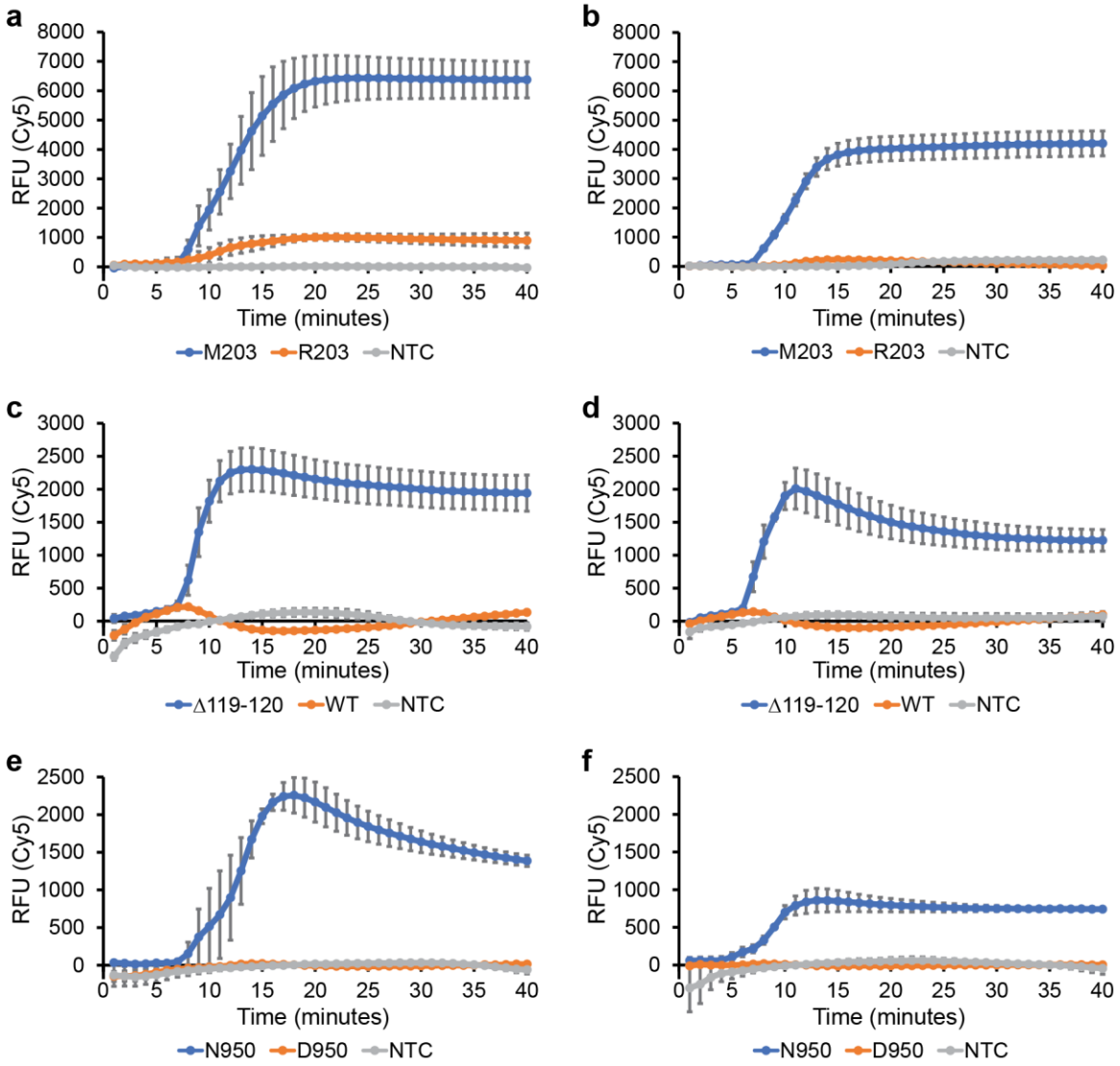
Supplementary Figure 22. Evaluation of various SNiPER probes for detection of A1708D in the ORF1ab gene. 2E4 copies of wildtype (A1708) or mutant (D1708) synthetic RNA fragments were used as template. Five probes were examined at 0.5µM concentration, namely **a** 16nt−LNA(3), **b** 15nt−LNA(3), **c** 15nt−LNA(1,3), **d** 15nt−LNA(2,3), and **e** 14nt−LNA(2,3). Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



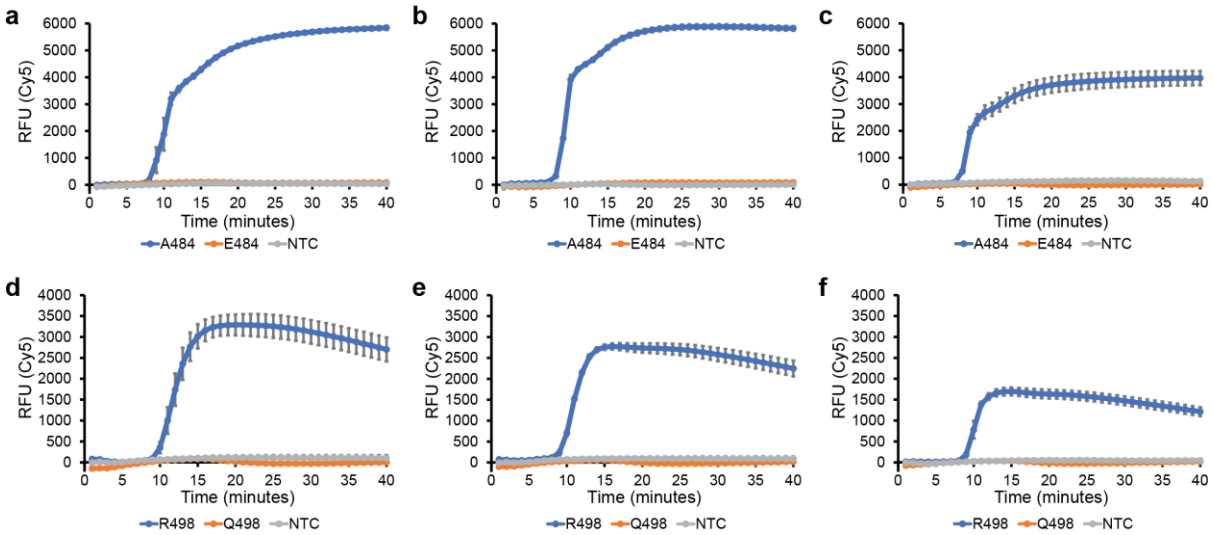
Supplementary Figure 23. Evaluation of D1708 SNiPER probes, namely **a** 15nt-LNA(1,3), **b** 15nt-LNA(2,3), and **c** 14nt-LNA(2,3), in the presence of Q5 high-fidelity DNA polymerase. The three probes were tested at 0.5µM concentration with 2E4 copies of wildtype (A1708) or mutant (D1708) synthetic RNA template. Every reaction mix also contained 0.25ng PC9 RNA and 1µM LANTERN probe against human ACTB. Fluorescence was monitored in the Cy5 channel (ORF1ab gene) and the HEX/SUN channel (ACTB), with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



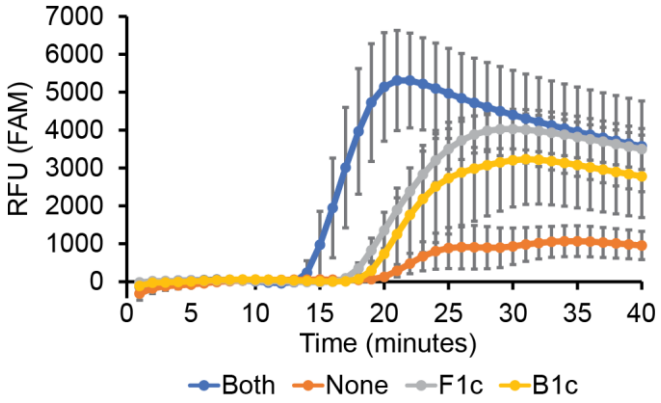
Supplementary Figure 24. Screening of multiple sets of LAMP primers for different Delta and Omicron mutations. Data represent mean ± s.e.m. (n = 3 [Δ119-120; R203M Combi1, Combi2, Combi3, Combi5, Combi6, and Combi8], 4 [R203M Combi4; D950N Combi1 and Combi2], or 5 [R203M Combi7; D950N Combi3; E484A/Q498R] biological replicates).



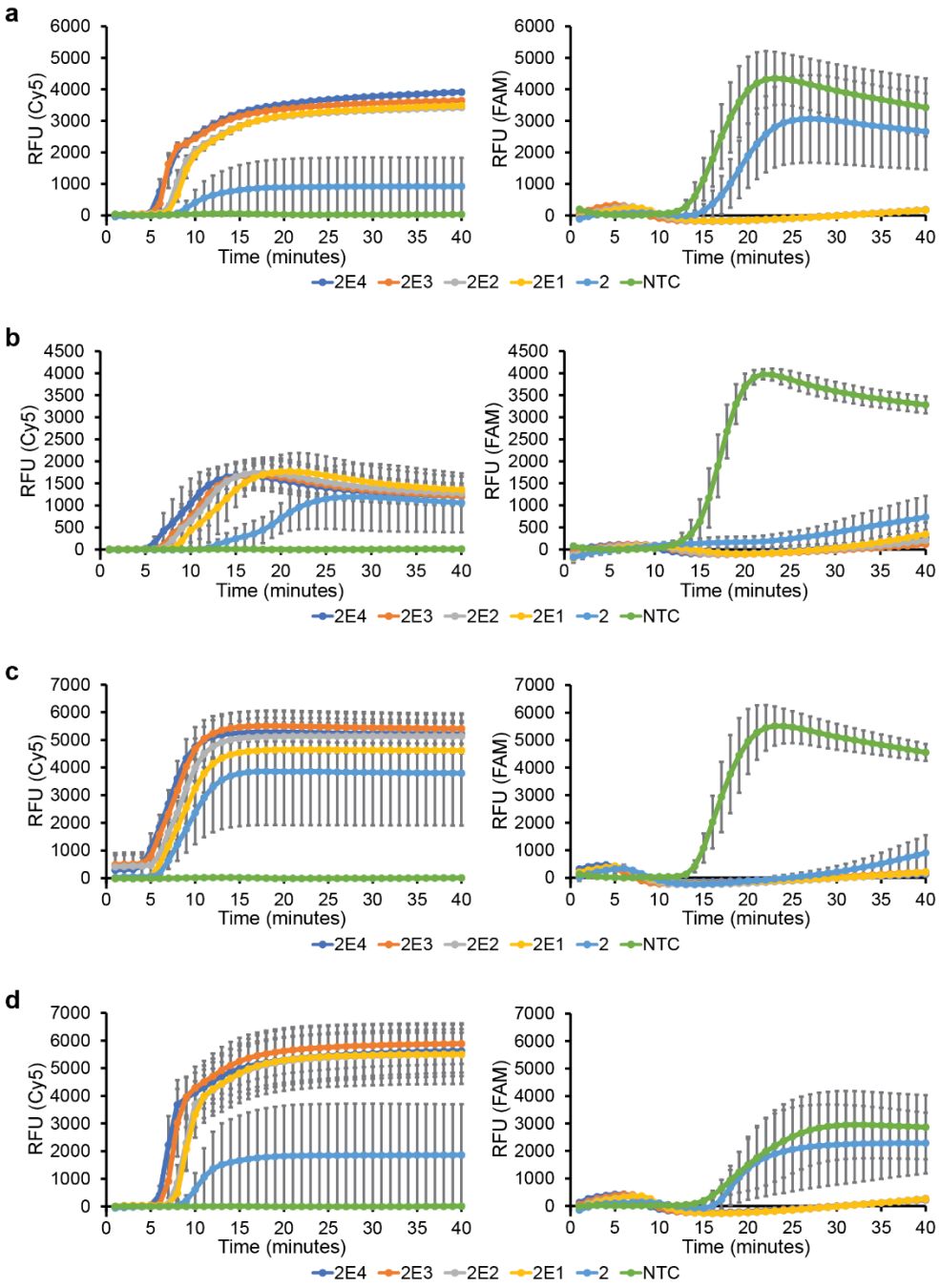
Supplementary Figure 25. Evaluation of various SNiPER probes for detection of different Delta mutations, namely R203M in the N-gene (**a** 15nt−LNA(2,3) and **b** 14nt−LNA(2,3)), Δ119-120 in the ORF8 gene (**c** 19nt−LNA(2,3) and **d** 18nt−LNA(2,3)), as well as D950N in the S-gene (**e** 19nt−LNA(2,3) and **f** 18nt−LNA(2,3)). 2E4 copies of the corresponding wildtype or mutant synthetic RNA fragments were used as template. The six probes were examined at 0.5µM concentration. Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 [R203M, D950N] or 4 [Δ119-120] biological replicates).



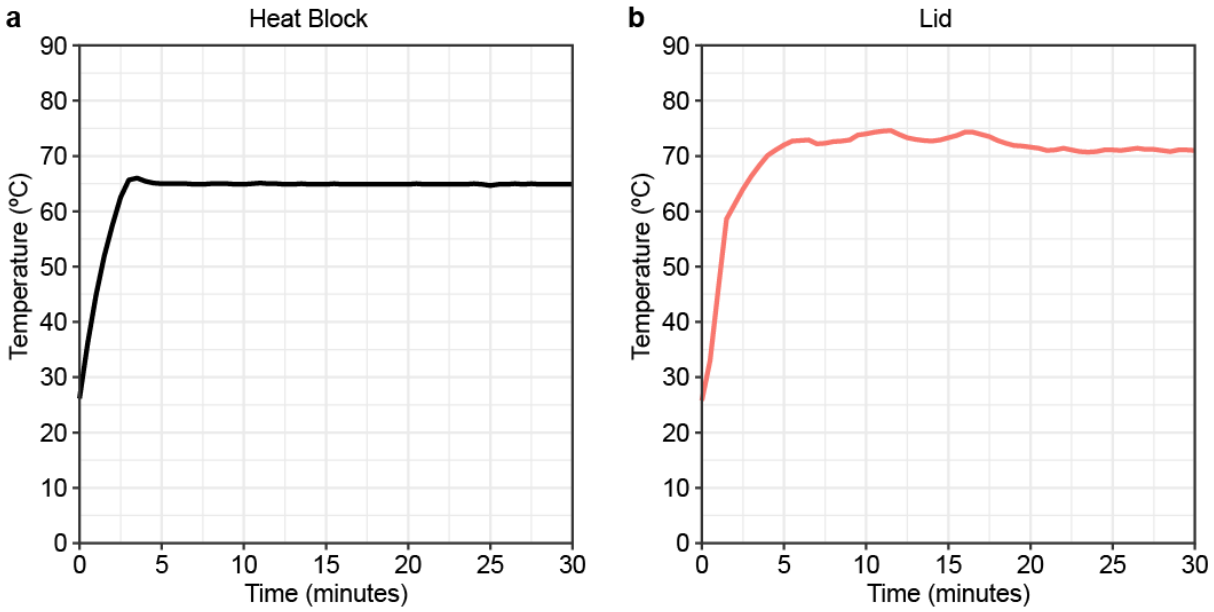
Supplementary Figure 26. Evaluation of various SNiPER probes for detection of different Omicron mutations, specifically E484A in the S-gene (**a** 20nt−LNA(2,3), **b** 19nt−LNA(2,3), and **c** 18nt−LNA(2,3)) as well as Q498R in the S-gene (**d** 17nt−LNA(2,3), **e** 16nt−LNA(2,3), and **f** 15nt−LNA(2,3)). 2E4 copies of the corresponding wildtype or mutant synthetic RNA fragments were used as template. The six probes were examined at 0.5µM concentration. Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 4 [E484A 20nt/19nt, Q498R] or 5 [E484A 18nt] biological replicates).



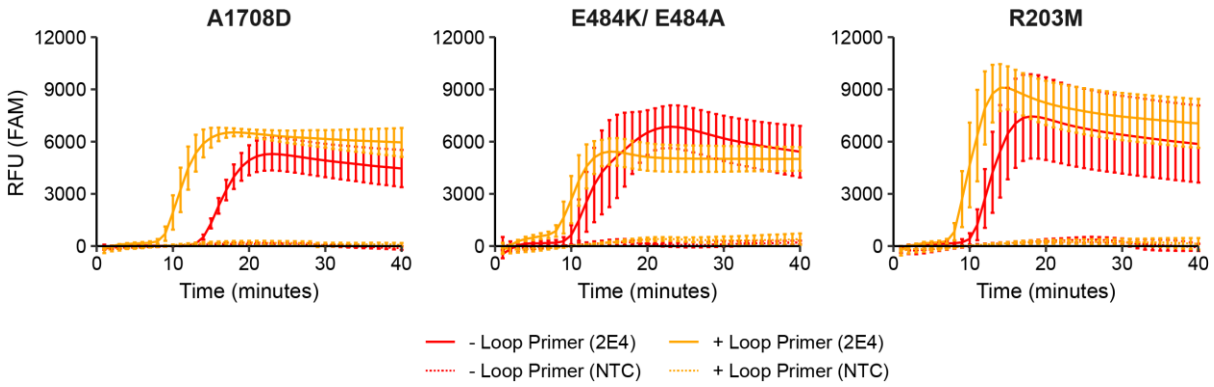
Supplementary Figure 27. Time courses showing the effect of one or two swarm primers on the amplification of the human ACTB gene. RT-LAMP reactions were supplemented with 8U of IsoPol+ and tested against 0.25ng PC9 RNA. Every reaction contained 0.5µM SNiPER probe targeting the LoopB region of the ACTB amplicon. Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 4 [both, none, B1c] or 8 [F1c] biological replicates).



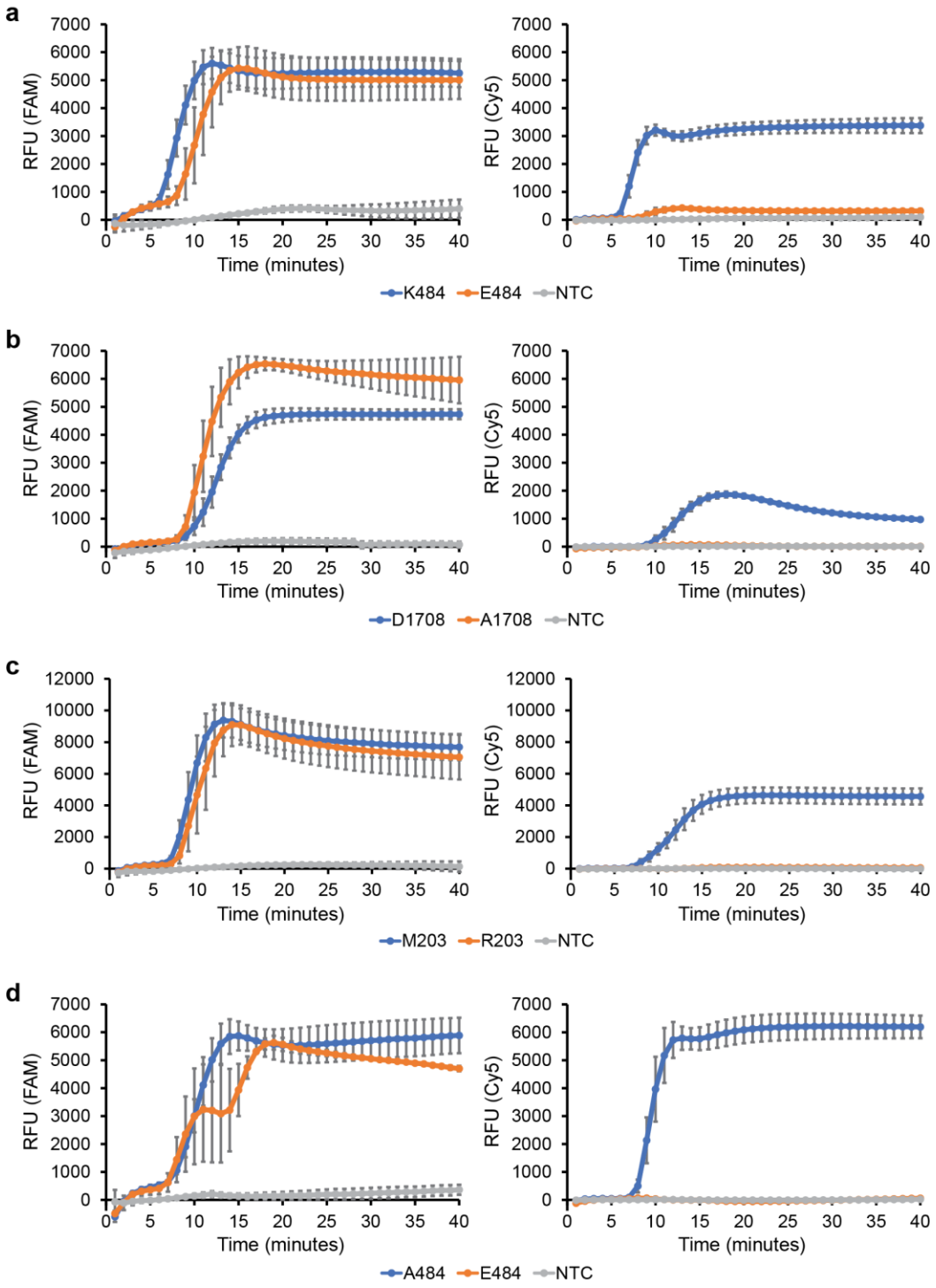
Supplementary Figure 28. Time courses of the fluorescence intensity in 2-plex SNiPER assays with human internal control. Variable copies of mutant synthetic viral RNA were added with 0.25ng of PC9 RNA as template. Every RT-LAMP reaction contained 0.5µM of SNiPER probe against ACTB and 0.5µM of SNiPER probe targeting the indicated mutation, specifically **a** E484K (Beta), **b** A1708D (Alpha), **c** R203M (Delta), and **d** E484A (Omicron). ACTB LAMP primers were added at 0.2X concentration with only one swarm primer (F1c). Each reaction was also supplemented with 8U of IsoPol+. Fluorescence was monitored in the Cy5 and FAM channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 [A1708D, R203M, E484A] or 4 [E484K] biological replicates).



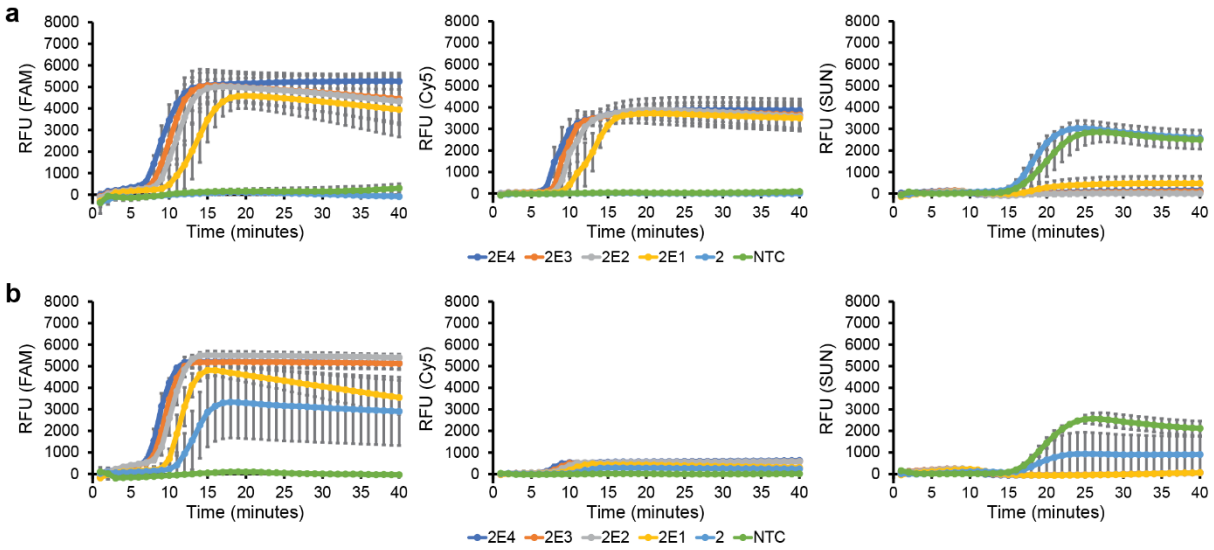
Supplementary Figure 29. Exemplary temperature measurements over time of the **a** heat block and **b** device lid using a thermistor. The device was able to reach the required temperature for RT-LAMP within 5 minutes of turning on the Peltier heater.



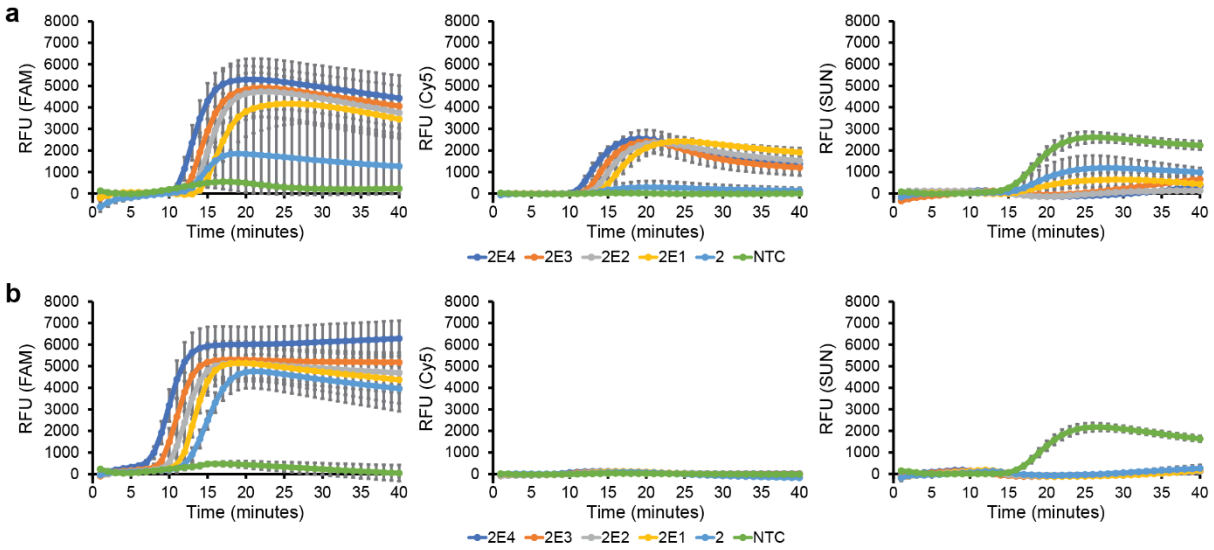
Supplementary Figure 30. Time courses for RT-LAMP reactions with or without a loop primer that may interfere with the corresponding SNiPER probe. Synthetic RNA templates matching the wildtype virus were used. Fluorescence was monitored in a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



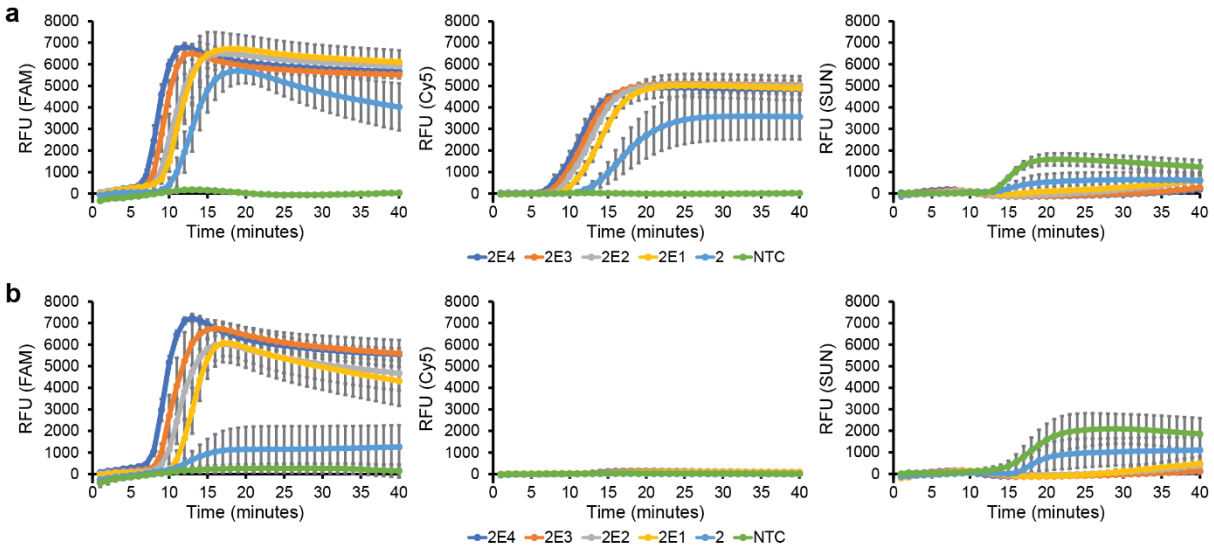
Supplementary Figure 31. Evaluation of 2-probe SNiPER assays for different SARS-CoV-2 variants, namely **a** Beta (E484K), **b** Alpha (A1708D), **c** Delta (R203M), and **d** Omicron (E484A). 2E4 copies of either mutant or wildtype synthetic RNA were used as template. Fluorescence was monitored in the FAM (loop probe) and Cy5 (SNP probe) channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 [E484K: E484 and K484 templates; A1708D: A1708 template; R203M], 4 [E484K: NTC; E484A: E484 and A484 templates], 5 [E484A: NTC], or 6 [A1708D: D1708 template and NTC] biological replicates).



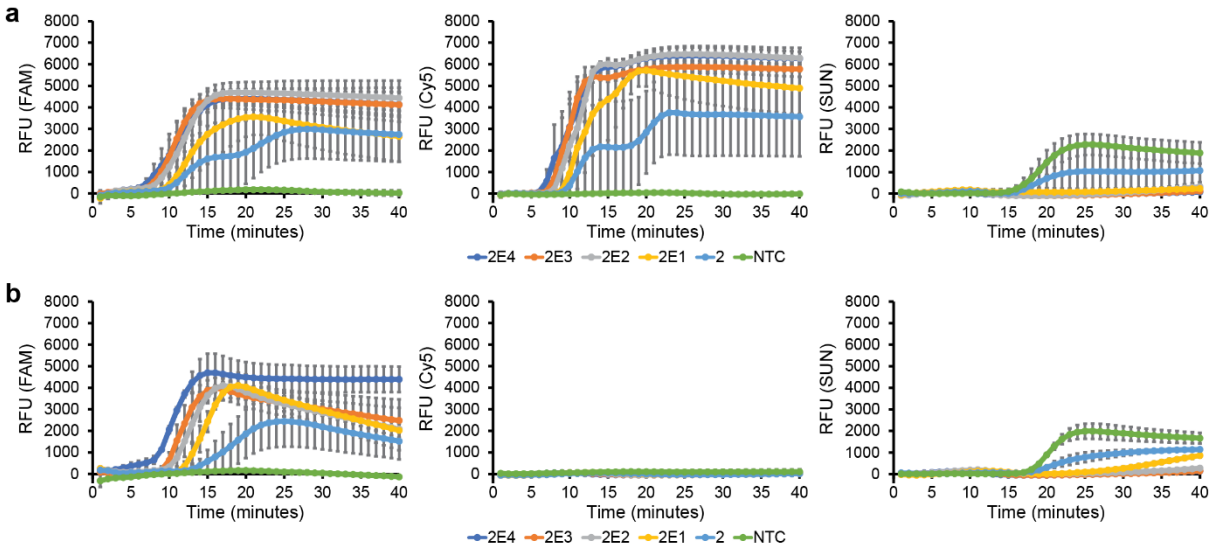
Supplementary Figure 32. Evaluation of a 2-plex 3-probe assay for the E484K mutation. Variable copies of **a** mutant (K484) or **b** wildtype (E484) synthetic viral template were spiked into 0.25ng human RNA from the PC9 cell line. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



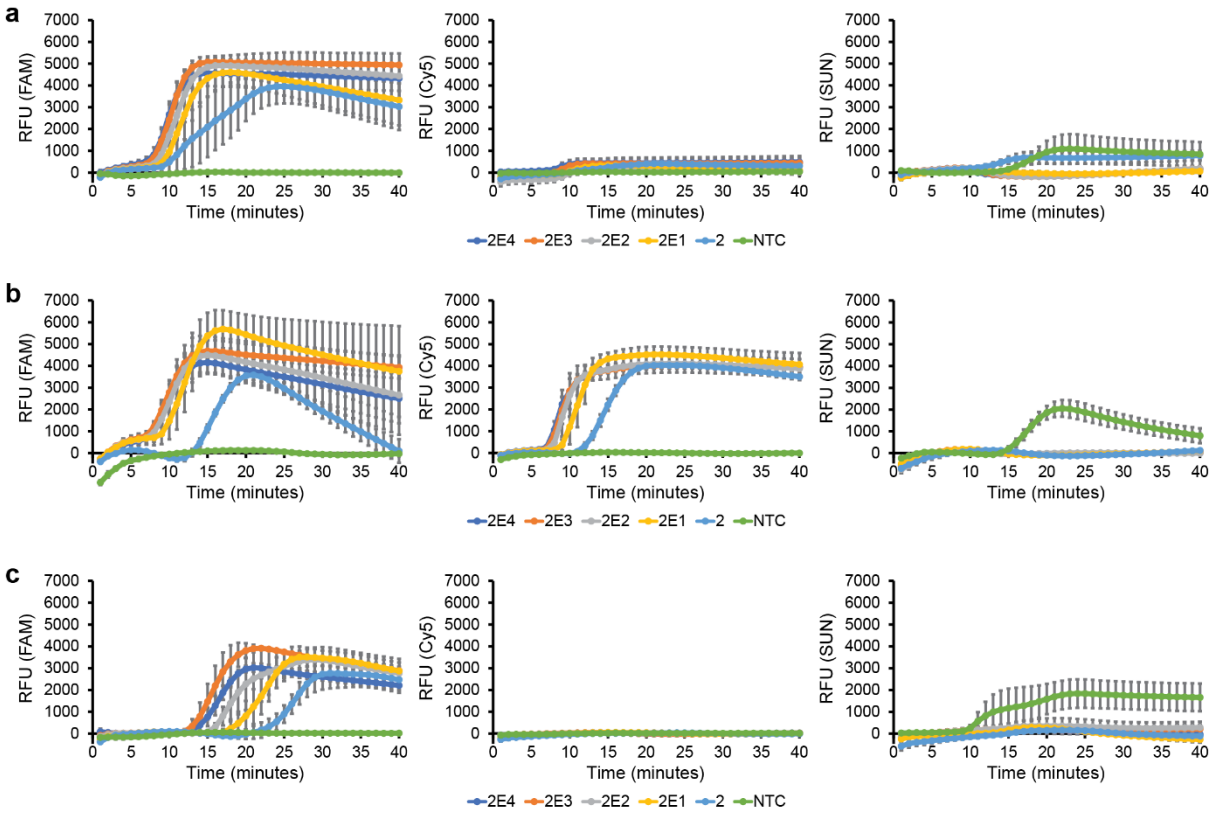
Supplementary Figure 33. Evaluation of a 2-plex 3-probe assay for the A1708D mutation. Variable copies of **a** mutant (D1708) or **b** wildtype (A1708) synthetic viral template were spiked into 0.25ng human RNA from the PC9 cell line. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



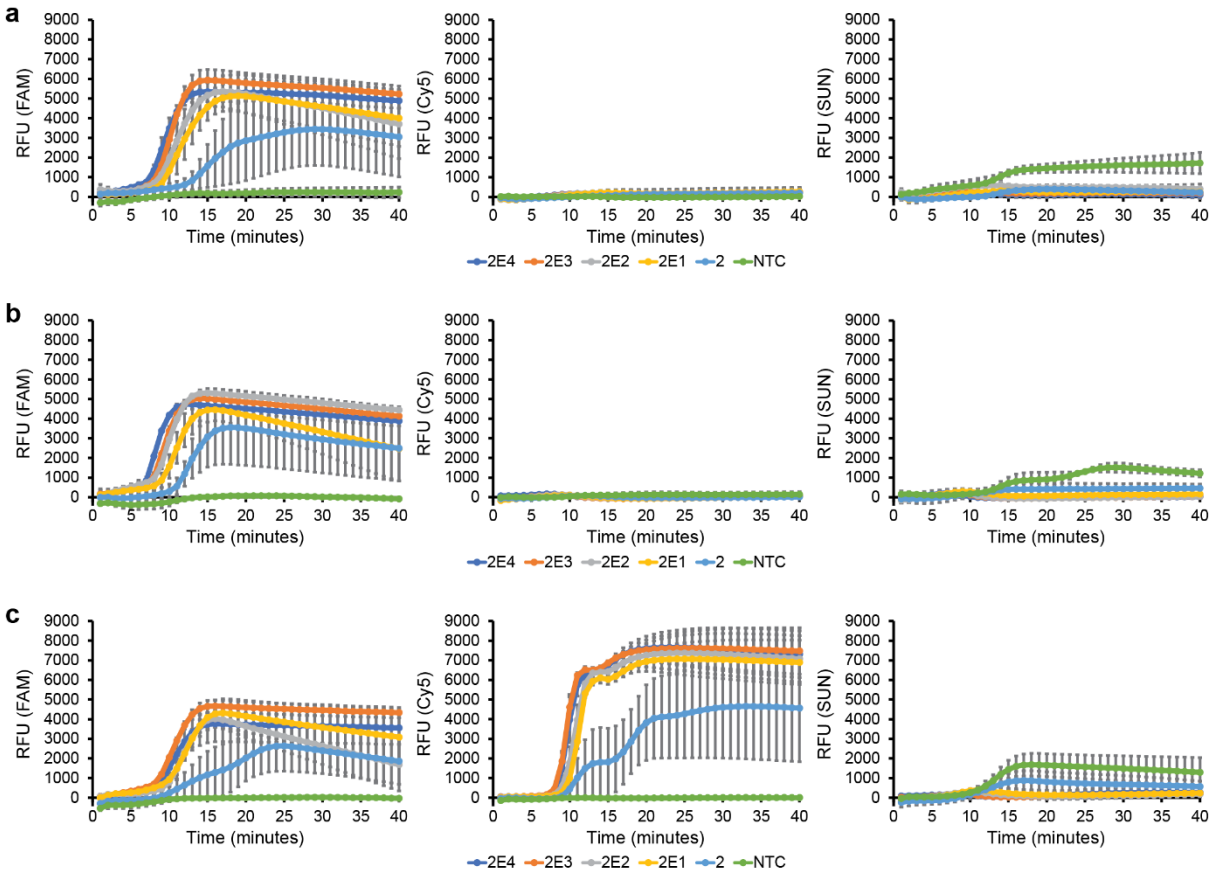
Supplementary Figure 34. Evaluation of a 2-plex 3-probe assay for the R203M mutation. Variable copies of **a** mutant (M203) or **b** wildtype (R203) synthetic viral template were spiked into 0.25ng human RNA from the PC9 cell line. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 2 [NTC for M203], 3 [2-2E4 for M203], 4 [NTC and 2-2E3 for R203], 5 [2E4 for R203 (FAM)], or 6 [2E4 for R203 (Cy5 and SUN)] biological replicates).



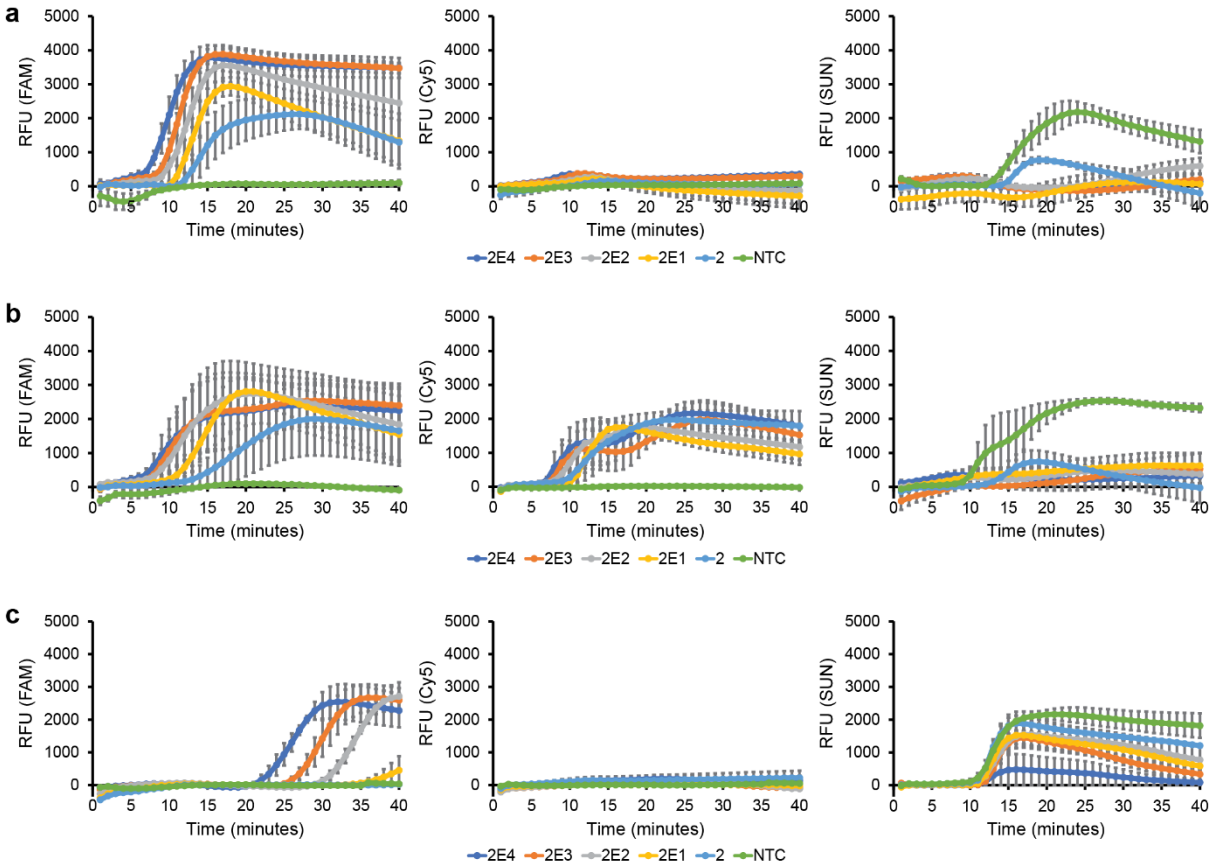
Supplementary Figure 35. Evaluation of a 2-plex 3-probe assay for the E484A mutation. Variable copies of **a** mutant (A484) or **b** wildtype (E484) synthetic viral template were spiked into 0.25ng human RNA from the PC9 cell line. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



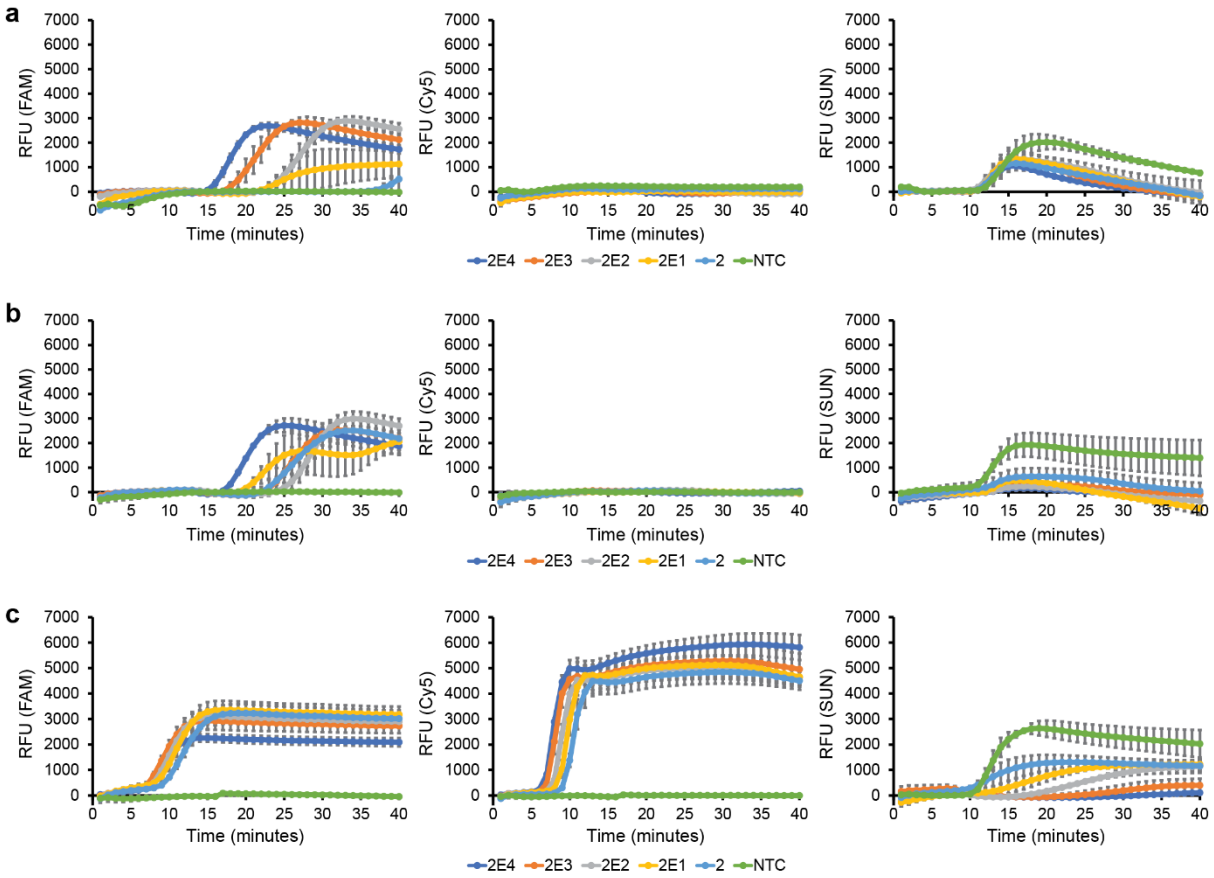
Supplementary Figure 36. Evaluation of 3-probe assays, with one of them being the K484 SNP probe. WarmStart LAMP reagents that had been stored frozen at -20°C were used on variable copies of **a** wildtype pseudovirus, **b** Beta pseudovirus, and **c** Omicron pseudovirus. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels of a real-time PCR instrument, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 [E484: NTC, 2E2-2E4; K484: 2-2E2; A484: 2E2-2E3], 4 [E484: 2-2E1; K484: NTC, 2E3-2E4; A484: NTC, 2E4], or 5 [A484: 2-2E1] biological replicates).



Supplementary Figure 37. Evaluation of 3-probe assays, with one of them being the A484 SNP probe. WarmStart LAMP reagents that had been stored frozen at -20°C were used on variable copies of **a** wildtype pseudovirus, **b** Beta pseudovirus, and **c** Omicron pseudovirus. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).

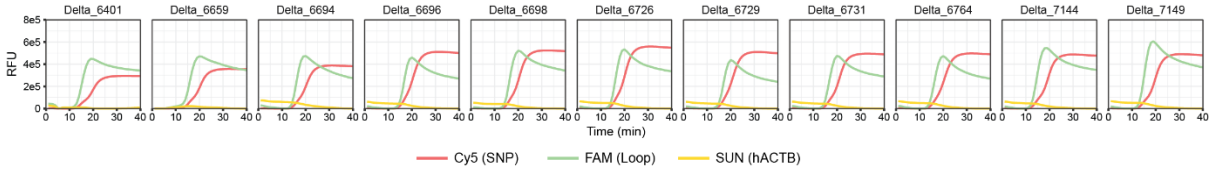


Supplementary Figure 38. Evaluation of 3-probe assays, with one of them being the K484 SNP probe. Lyophilized LAMP reagents that had been stored at room temperature were used on variable copies of **a** wildtype pseudovirus, **b** Beta pseudovirus, and **c** Omicron pseudovirus. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).



Supplementary Figure 39. Evaluation of 3-probe assays, with one of them being the A484 SNP probe. Lyophilized LAMP reagents that had been stored at room temperature were used on variable copies of **a** wildtype pseudovirus, **b** Beta pseudovirus, and **c** Omicron pseudovirus. Fluorescence was monitored in the FAM, Cy5, and HEX/SUN channels, with measurements taken every minute. Data represent mean ± s.e.m. (n = 3 biological replicates).

Supplementary Figure 40. Sequence of the targeted region of the SARS-CoV-2 S-gene, with LAMP primer binding sites indicated in colour.



Supplementary Figure 41. Clinical evaluation of our Delta SNiPER assay. The Delta SNP probe correctly detected the presence of the Delta variant in all 11 samples.