Supplementary Information

Diagnostic performance of face masks for collection and detection of SARS-CoV-2

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Fig. S1 Process of isolating and extracting the virus-collected layer from the face mask. (a) Process of separating the filter layer from the face mask. (b) Schematic of the membrane layer of a face mask. (c) Overall process of RNA extraction from the collected mask.

Fig. S2 Method of increasing the amount of recovery of viral nucleic acids from a filter layer. (a)–(d) Proposal for maximal recovery of the mask lysis solution from the filter layer. (e) Amount of recovery of the lysis solution using a syringe and centrifuge. 2(f) Comparison of Ct values by quantifying nucleic acids targeting exosome genes using RT-qPCR.

Fig. S3 SARS-CoV-2 detection using DNA hydrogel formation. (a) COVID-19 non-pathogen (negative control), (b) N gene, (c) E gene, and (d) RdRp gene.

Fig. S4 Quantitative trend analysis using SARS-CoV-2 target gene according to the mask collection date. (a) E gene, (b) RdRp gene, and (c) N gene.

Fig. S5 Quantitative trend analysis using SARS-CoV-2 target gene according to each sample type. (a) E gene, (b) RdRp gene, and (c) N gene.

Fig. S6 Verification of hydrogel formation according to RCA reaction time. (a) 1 h, (b) 3 h, (c) 6 h, (d) 12 h, (e) 18 h, (f) 24 h, (g) 48 h, and (h) 72 h.

Fig. S7 Electrophoresis of non-specific reaction to synthesized viral oligo (Specificity verification)

Table S1 List of SARS-CoV-2 template, pathogen, linker primer, and primer nucleic acid sequences used in this study

Table S2 Diagnostic criteria by flow in RCA-flow system.

Materials

Pathogen nucleic acid sequences (26 nt) for COVID-19 (E, N, RdRp, ORF1ab gene); template DNAs (102 nt) for COVID-19 (E, N, RdRp, ORF1ab gene); primers (NH_2-polyA9-primer (NH2 primer: 32 nt), and additional primers (12 nt) were synthesized by Bioneer (Daejeon, Korea) (Table S1). Rigid Teflon tubes (ID = 0.4 mm, OD = 0.9 mm) and flexible silicon tubes (ID = 0.8 mm, OD = 1.8 mm) were purchased from Sungjin Rubber Co. (Seoul, Korea). Nylon mesh with a 1-μm pore was purchased from ELKO Filtering Co. (Florida, USA). Bst 3.0 DNA polymerase, 10× Isothermal Amplification Buffer II, T7 ligase, and pyrophosphatase were obtained from New England Biolabs (Ipswich, MA, USA). A total of 25 mM dNTP was obtained from Thermo Fisher Scientific Inc. (MA, USA) and 100 mM DTT was purchased from Epi-center Technologies Corp. (Madison, USA). Bovine serum albumin (BSA) and adenosine triphosphate (ATP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The amine coupling kit (A515-10: NHS buffer, WSC buffer, activation buffer, reaction buffer, and blocking buffer) was purchased from Dojindo (Kumamoto, Japan). A T100 thermal cycler was purchased from Bio-Rad (Hercules, CA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immobilization of Linker Primer on Nylon Mesh Surface

After the washing step, 200 μL of 100-mM NHS buffer and 200 μL of 100-mM WSC buffer (both from the coupling kit) were mixed with nylon mesh and incubated at room temperature (~23 °C) for 30 min. The nylon mesh was then washed twice with 500 μL of 1×PBS buffer. The washed nylon mesh was then resuspended in 380 μL of reaction buffer and 20 μL of 1-mM NH2 primer and incubated for 2 h. The nylon mesh was then washed twice with 500 μL of 1×PBS buffer, resuspended in 500 μL of blocking buffer and incubated for 1 h. The nylon mesh was washed twice and resuspended in 400 μL of 1×PBS buffer.

Extraction of SARS-CoV-2 virus nucleic acid from face mask

The process of extracting RNA from the mask was as follows. First, the mask membrane was cut to the size required for sample extraction. 5 ml of Trizol was dispensed on the membrane of the mask, incubated at room temperature for 10 min, and then the entire solution was drained from the mask using ultracentrifuge (7000 g, 15 min). 200 µl of chloroform was dispensed into 1.5 tubes, and 1 ml of the mixture separated from the mask was added. Subsequently, it was shaken for 15 s and incubated at room temperature for 5 min. After centrifuging at 12,000 g, 4 °C, 10 min, we separated only the transparent supernatant. After mixing 500 µl of isopropanol with the supernatant, we incubated it for 10 min at room temperature after stirring. It was centrifuged again at 12,000 g, 4 °C, 10 min, and then the supernatant was removed. After adding 1 ml of 75% ethanol to the RNA from which the supernatant was removed, we it centrifuged at 7500 g, 4 °C, 5 min, and then removed the supernatant (ethanol). It was dried (to remove ethanol completely) for 5–-10 min with the lid open, and washing was performed twice depending on the sample. Finally, after dispensing 15 µl of RNase-free water (DW) and pipetting, we incubate it at 65 °C for 10 min in a heating block, and then incubate it on ice for 2 min to extract RNA.

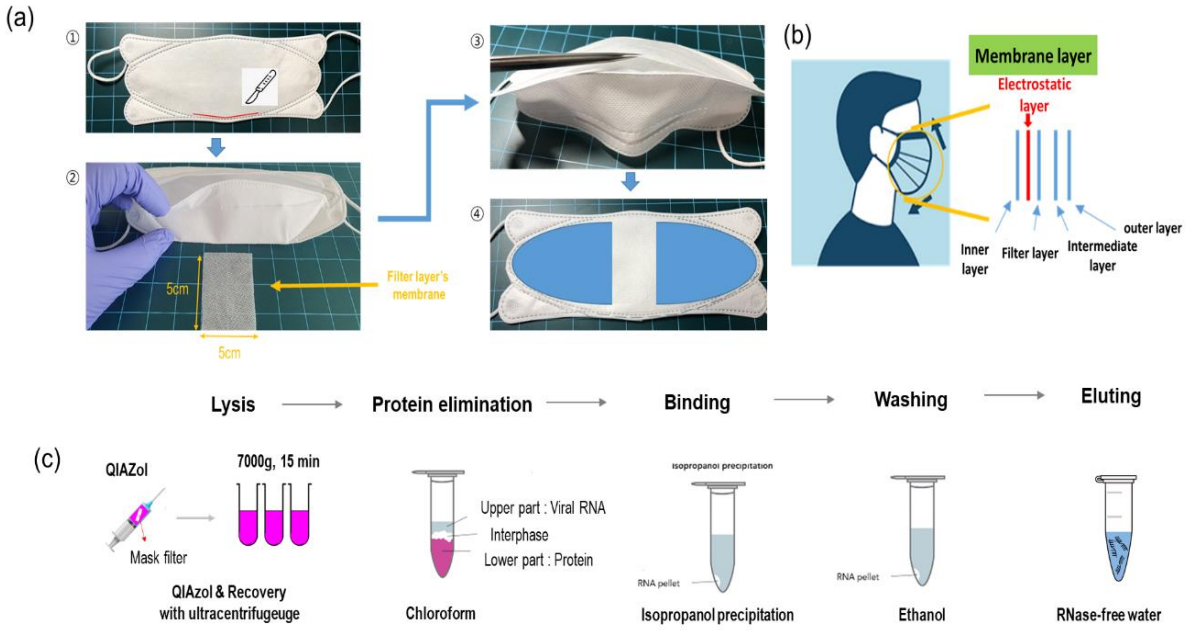

Fig. S1 Process of isolating and extracting the virus-collected layer from the face mask. (a) Process of separating the filter layer from the face mask; it is useful to use all possible filter membranes (5 cm ×5 cm) in terms of the amount of virus RNA extracted. (b) Schematic of the membrane layer of a face mask; the face mask consists of four layers (outer, intermediate, filter, and inner layers). (c) Overall process of RNA extraction from the collected mask.

Increase in the amount of recovery of viral nucleic acids from the filter layer

A centrifuge, not a syringe, was used to increase the recovery of viral nucleic acids from the filter layers. First, the mask membrane was cut to the required size (Fig. S2 (a) to (d)). After incubating the mask membrane in 5 ml of Trizol for 10 min at room temperature, it was placed into the body of the 10 ml syringe. Next, the syringe body was increased by inserting it into the cap of the 50 ml tube, and the combined cap was inserted into the 50 ml tube and locked. The entire solution was then recovered from the mask using an ultracentrifuge (7000 g, 15 min). When using 5 ml of lysis buffer in the mask, 4–4.4 ml (80%–86%) of the solution could be recovered with a syringe, but more than 4.8 ml (95%) of the solution could be recovered when using a centrifuge. the protocol for the extraction method is also important for increasing the concentration to improve the virus concentration problem. However, the concentration may also increase when the recovery rate of the mask lysis solution is raised to 95% or more. As shown in Fig. S2 (e), we confirmed the amount of recovery of the lysis solution using a syringe and centrifuge. The centrifugation method had a relatively large amount of recovery. Moreover, as shown in S2(f), using this sample, we could quantify nucleic acids targeting exosome genes using RT-qPCR and compare their Ct values. The centrifuged samples were observed to be relatively high in concentration.

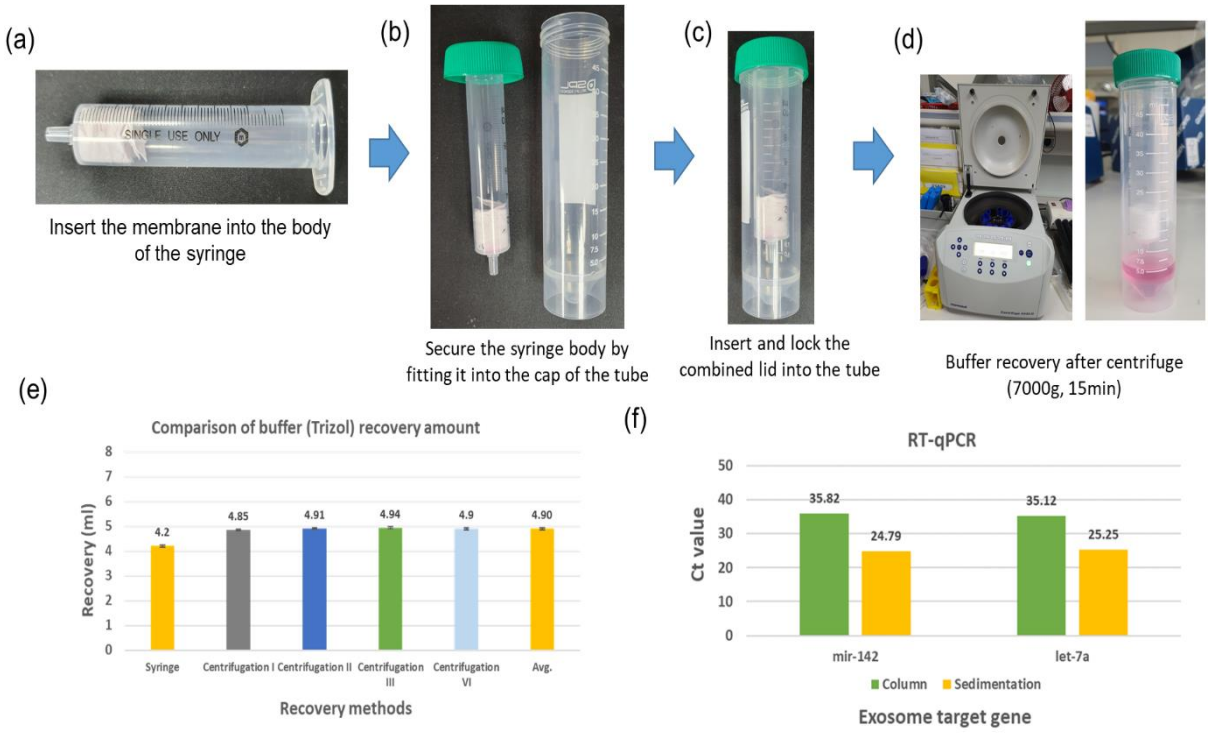

Fig. S2 Method of increasing the amount of recovery of viral nucleic acids from a filter layer. (a)–(d) Proposal for maximal recovery of the mask lysis solution from the filter layer. (e) Amount of recovery of the lysis solution using a syringe and centrifuge. 2(f) Comparison of Ct values by quantifying nucleic acids targeting exosome genes using RT-qPCR.

Validation of DNA hydrogel formation by SARS-CoV-2 templates

We obtained 1 μL of 100 μM clinical mask sample (Nucleic acid, RNA), 1 μL of T4 ligase (400 U/μL), 1 μL of pyrophosphatase (100 U/mL), 4 μL of phi29 DNA polymerase (10 U/μL), 10× phi29 40 μL consisting of 4 μL of polymerase buffer, 1 μL of 100 mM DTT, 4 μL of 25 mM dNTP, 1 μL of 50 mM ATP, 1 μL of 100 μM additional primer, 1 μL of BSA (10 mg/mL) and 21 μL of distilled water. We prepared the RCA mixture. To confirm the formation of DNA hydrogels, we suspended several types of SARS-CoV-2 templates in the RCA mixture and incubated at 30 °C for 60 min.

Owing to the dumbbell-shaped template, amplified long DNAs tend to easily entangle, aggregate with neighboring DNAs, and form a DNA hydrogel. After incubation, sufficient hydrogel formation was induced in the tube to which the N, E, and RdRp gene templates of SARS-CoV-2 were linked. In contrast, no DNA hydrogel was formed in the tube without SARS-CoV-2 template. The captured image (Fig. S3) indicated that a strong DNA hydrogel was formed depending on the target template.

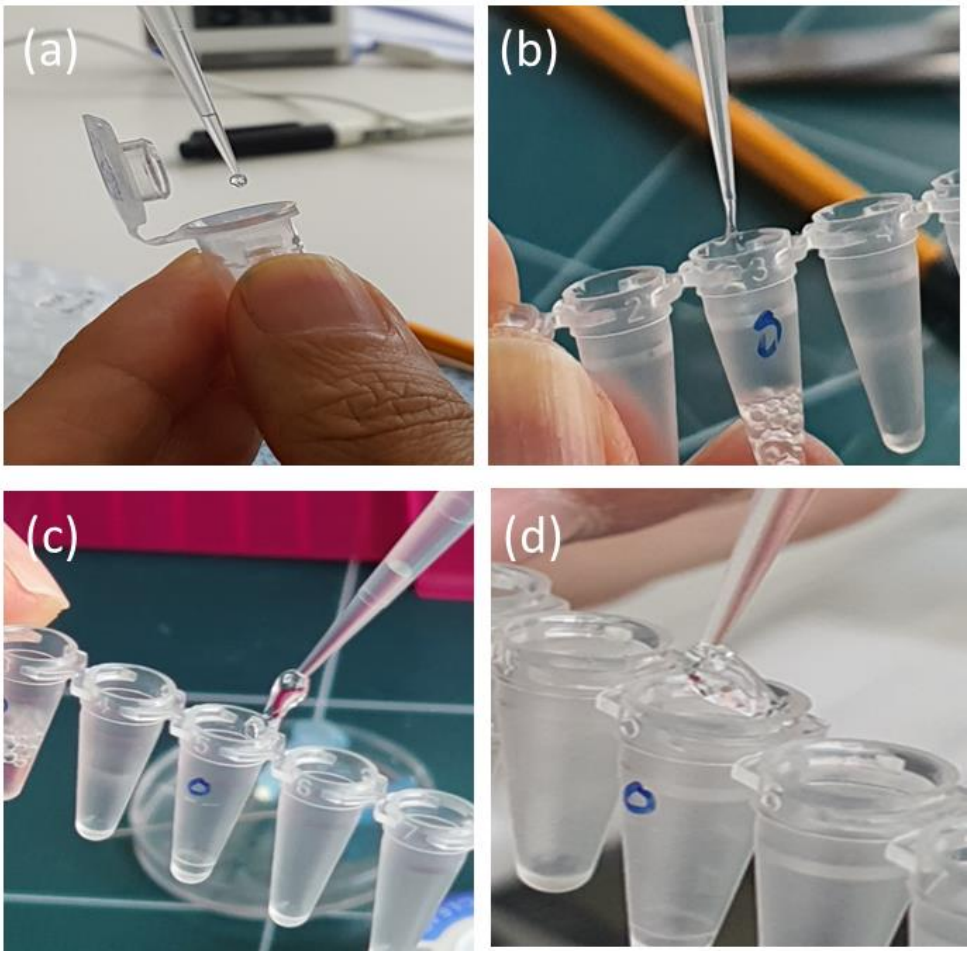

Fig. S3 SARS-CoV-2 Detection using DNA hydrogel formation. (a) COVID-19 non-pathogen (negative control), (b) N gene, (c) E gene, and (d) RdRp gene.

Comparison of quantitative values for each SARS-CoV-2 target gene according to the date of mask collection

Analysis was performed using N=9 masks collected from the first to the third day of the collection day. Quantification was performed using RT-PCR, and the SARS-CoV-2 target gene (E, RdRp, and N genes) were targeted. As shown in Fig. S4 (a) to (c), the total Ct value for each target gene tended to be pushed back; therefore, we confirmed that the amount of virus tended to decrease after the collection day passed.

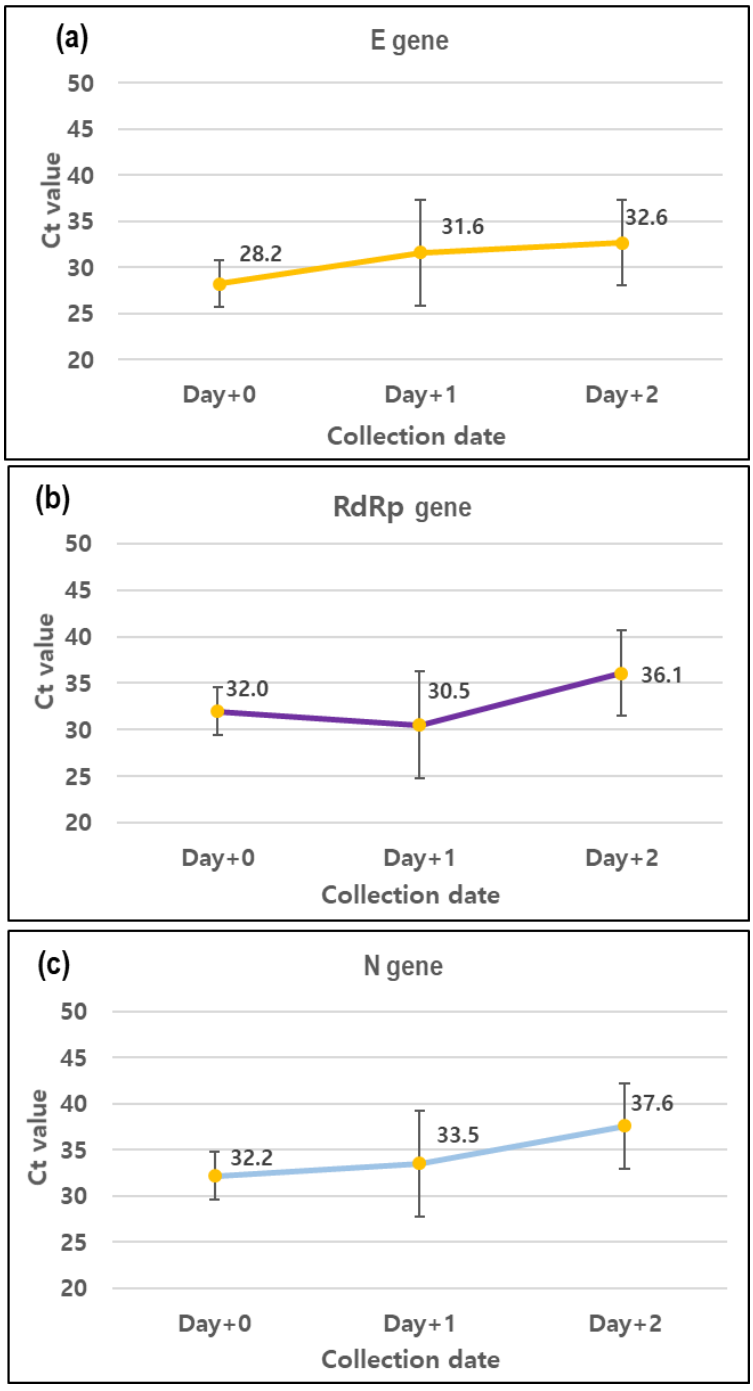

Fig. S4 Quantitative trend analysis by SARS-CoV-2 target gene according to the mask collection date. (a) E gene, (b) RdRp gene, and (c) N gene.

Comparison of quantitative values for SARS-CoV-2 target gene according to each sample

Analysis was performed using each N=3 sample according to the sample type (nasal swap, saliva, mask). Virus quantification was performed using RT-PCR, and SARS-CoV-2 target genes (E, RdRp, N genes) were used.

As shown in Fig. S5 (a) to (c), no significant difference was observed in the Ct value for each target gene depending on the type of sample. Overall, we observed that the performance of collecting nucleic acids from the mask is not significantly inferior to that used for conventional sample collection.

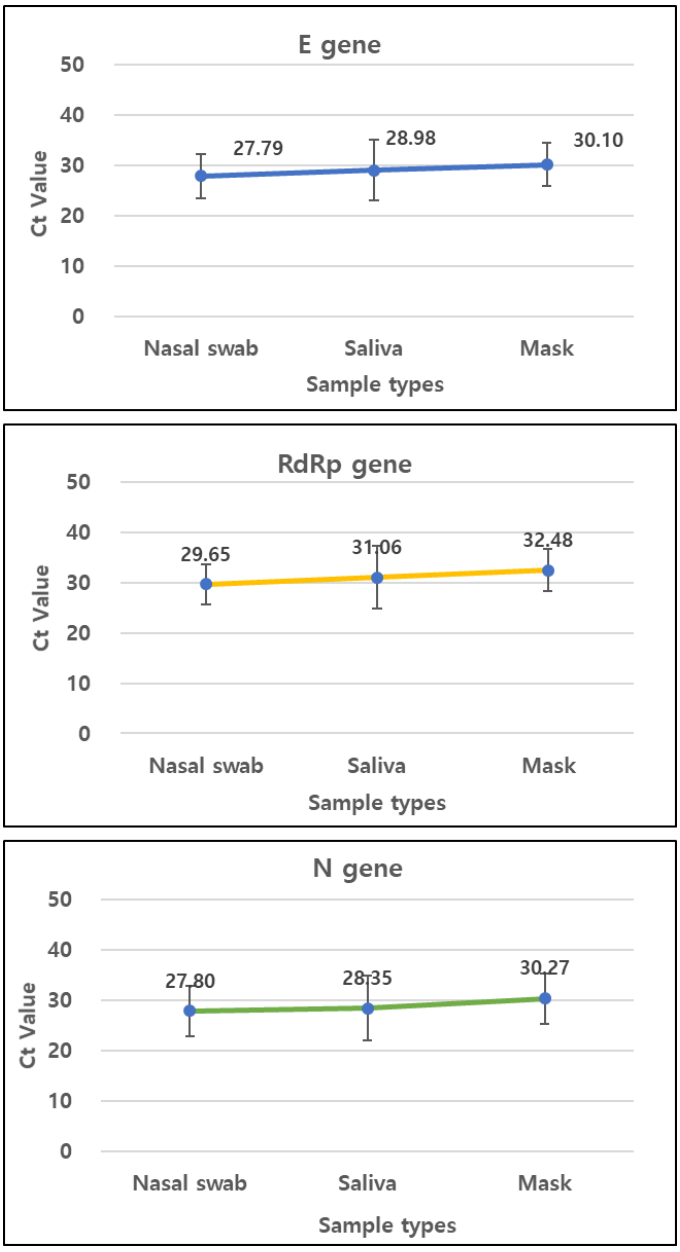

Fig. S5 Quantitative trend analysis by SARS-CoV-2 target gene according to each sample type. (a) E gene, (b) RdRp gene, and (c) N gene.

Verification of false-positive pattern according to RCA reaction time

Using the padlock probe (template) for the E gene of COVID-19 (SARS-CoV-2), the induction of a false positive reaction for the E gene and RdRp gene was confirmed on the tube. As shown in the results of Fig. S6 (a)–(h), first, for a positive, it can be seen that in Fig. S6(a), a band of the hydrogel was formed gradually, which increased according to the RCA incubation time. However, fortunately, it increased up to 72 h, but a tendency to decrease after a certain period was confirmed.

Next, we confirmed that hydrogel formation did not occur at all in the experimental group that did not include a template. This was because the included target was short and dimer formation was not performed with this. We could consider that the main factor in dimer formation was the template.

In the experimental group not including the target, hydrogel formation did not occur until the time of Fig. S6 (d), but it was formed gradually from Fig. S6 (e). Thus, the increase in hydrogel formation with time in the untargeted experimental group implies that the template was ligated, which was considered a false positive sign. Finally, in the test group with a template, but with a different target, hydrogel was clearly formed from Fig. S6 (g), but rather decreased from Fig. S6 (h). In conclusion, the false-positive response could have been induced by the template (102 nt) rather than the target (26 nt). The ligation of the templates most likely resulted from dimer formation between the templates. Therefore, to reduce false-positive reactions, it will be necessary to supplement the template by blocking the base dimer formation, changing the concentration, and modifying a part of the base sequence.

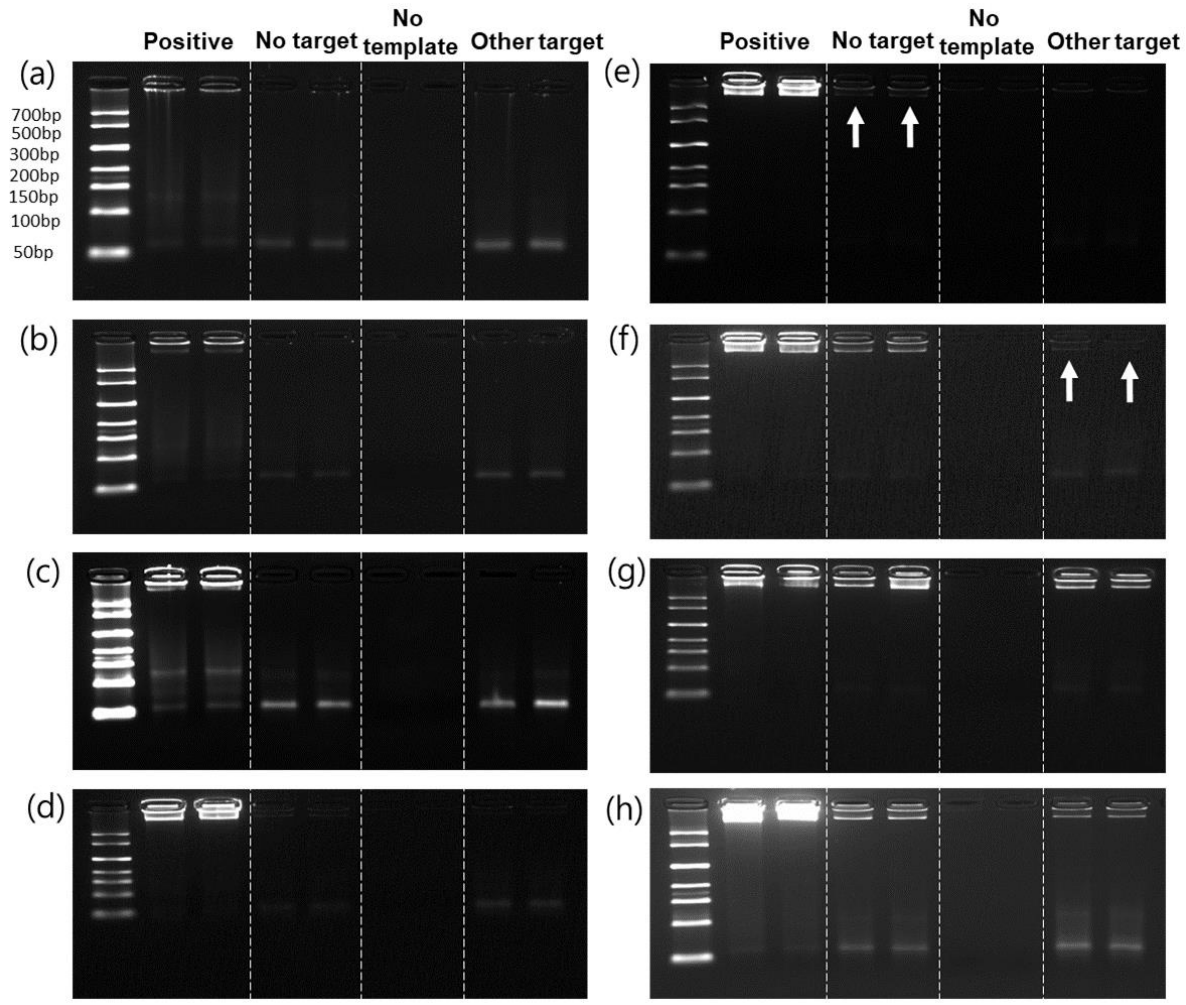

Fig. S6 Verification of hydrogel formation according to RCA reaction time. (a) 1 h, (b) 3 h, (c) 6 h, (d) 12 h, (e) 18 h, (f) 24 h, (g) 48 h, and (h) 72 h.

Validation of non-specific responses to RCA-based false-positive

Specificity verification for COVID-19 was confirmed on the tube using the synthesized viral oligos. The RCA reaction time was designated as 3 h by dedicating sufficient time (considering false-positive induction) than the existing virus detection time.

For the template, a probe conjugated to the RdRp gene was used. In response, several viral oligos and RCA reactions were induced. As shown in Fig. S7, the RCA reaction was induced only for the matched target, and the product, hydrogel, was formed. Hydrogel was not formed in other targets, verifying that there was no problem in specificity, and the same experiment was performed using viruses and plasmids, in addition to the above synthetic oligos.

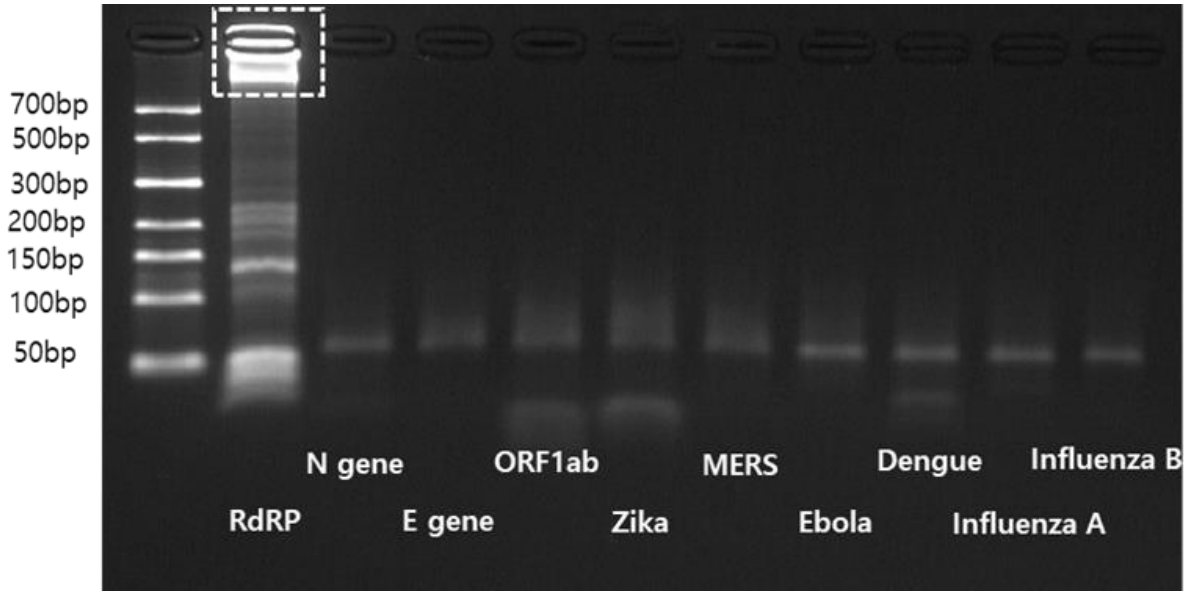

Fig. S7 Electrophoresis of non-specific reaction to synthesized viral oligo (specificity verification).

Table S1. List of SARS-CoV-2 template, pathogen, linker primer, and primer nucleic acid sequences used in this study.

Strands	Sequence (5' → 3')
COVID-19 N gene template (102 nt)	5'-phosphate-AAT ACC ATC TT A ATC GAA GTA CTC AGC GTA AGT TTA GAG GTA GCA TGC TAG TAT CGA CGT CCC ACG TAC CAA CTT ACG CTG AGT ACT TCG ATT GGT AGT AGA-3'
COVID-19 E gene template (102 nt)	5'-phosphate-CAC GTT AAC AAA ATC GAA GTA CTC AGC GTA AGT TTA GAG GTA GCA TGC TAG TAT CGA CGT CCC ACG TAC CAA CTT ACG CTG AGT ACT TCG ATT TAC AAG ACT-3'
COVID-19 RdRp gene template (102 nt)	5'-phosphate-GAA CTT CCT TCA ATC GAA GTA CTC AGC GTA AGT TTA GAG GTA GCA TGC TAG TAT CGA CGT CCC ACG TAC CAA CTT ACG CTG AGT ACT TCG ATT AAT TCA ACA-3'
COVID-19 ORF1ab gene template (102 nt)	5'-phosphate-GTA GCC ATA CTA ATC GAA GTA CTC AGC GTA AGT TTA GAG GTA GCA TGC TAG TAT CGA CGT CCC ACG TAC CAA CTT ACG CTG AGT ACT TCG ATT AAG TAG TAT-3'
COVID-19 pathogen(26 nt)_ N gene	5'- TAA AAG ATG GTA TTT CTA CTA CCT TA-3'
COVID-19 pathogen(26 nt)_ E gene	5'- TAA TTG TTA ACG TGA GTC TTG TAT TA-3'
COVID-19 pathogen(26 nt)_ RdRp gene	5'- TAA GAA GGA AGT TCT GTT GAA TTT TA-3'
COVID-19 pathogen ORF1ab (26 nt)	5'- TAA AGT ATG GCT ACA TAC TAC TT TTA-3'
NH2-polyA-primer (32 nt)	5'-Amino(C6)-AAA AAA AAA GGG ACG TCG ATA CTA GCA TGC TA-3'
Additional Primer (12 nt)	5'-TGC TAG TAT CGA-3'

*Red indicates the pathogen binding site, green indicates the self-assembly region, and blue indicates the primer binding site.

Table S2. Diagnostic criteria by flow in RCA-flow system.

Flow	Result
Block	Detected(+)
Touch down ≥ 20 s	
Touch down ≤ 20 s	Not detected(-)