Electronic Supplementary Material (ESI) for Analytical Methods.

Appendix A.

Supplementary material

Clinical evaluation of an innovative isothermal amplification detection system for COVID-19 diagnostics

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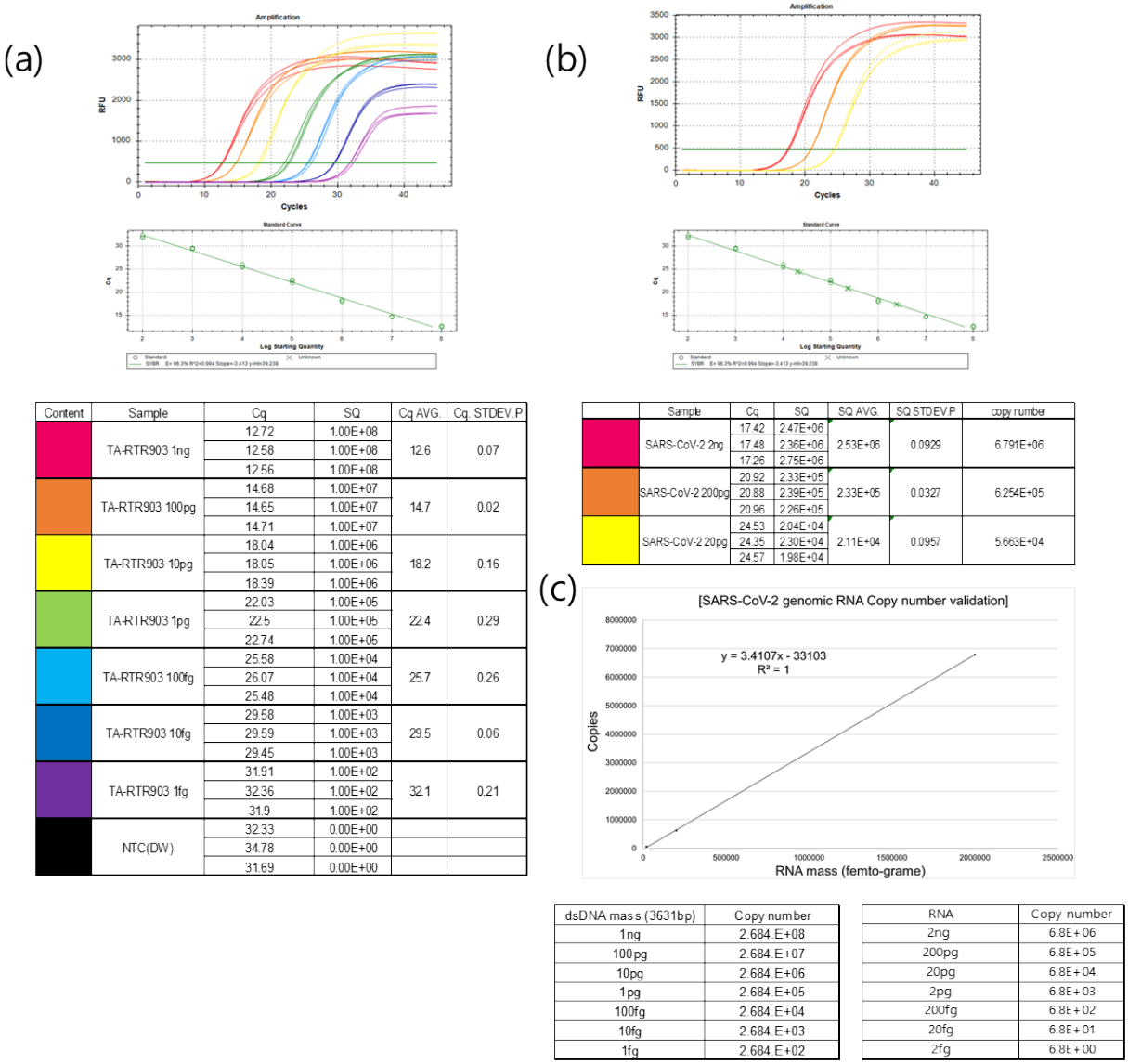
Preparation of lateral flow strip

The streptavidin (20 μL, 1 mg/mL) was added to a mixture of 1 mL of AuNP colloid (40 nm in diameter) and 100 μL of 0.1 M borate buffer (pH 8.5). After incubation for 1 h at 25 °C, 20 μL of 10% Bovine serum albumin in PBS was added to the mixture to block non-specific binding on the AuNP surface. After incubation for 1 h at 25 °C, the mixture was centrifuged at 9000 rpm for 15 min at 10 °C. The supernatant was discarded and the AuNP conjugate was resuspended in 0.1 mL of 10 mM borate buffer (pH 8.5). To prepare the lateral flow strip, 1 mg/mL Biotin–BSA and 1 mg/mL streptavidin were immobilized on an NC membrane (1 μL/cm) to create control line and test line, respectively. An absorbent pad was attached on top of the membrane. The AuNP conjugate was applied to the conjugate pad. The AuNP conjugate-loaded conjugate pad was attached to the bottom of the prepared strip, and a sample pad was placed underneath it to load the sample. The assembled membrane was cut into 4.0mm wide strips and stored in a humidity-controlled chamber (25 °C; 30% relative humidity) until use.



Content	Sample	Cq	SQ	Cq AVG.	Cq. STDEV.P
	TA-RTR903 1ng	12.72	1.00E+08	12.6	0.07
		12.58	1.00E+08		
		12.56	1.00E+08		
	TA-RTR903 100pg	14.68	1.00E+07	14.7	0.02
		14.65	1.00E+07		
		14.71	1.00E+07		
	TA-RTR903 10pg	18.04	1.00E+06	18.2	0.16
		18.05	1.00E+06		
		18.39	1.00E+06		
	TA-RTR903 1pg	22.03	1.00E+05	22.4	0.29
		22.5	1.00E+05		
		22.74	1.00E+05		
	TA-RTR903 100fg	25.58	1.00E+04	25.7	0.26
		26.07	1.00E+04		
		25.48	1.00E+04		
	TA-RTR903 10fg	29.58	1.00E+03	29.5	0.06
		29.59	1.00E+03		
		29.45	1.00E+03		
	TA-RTR903 1fg	31.91	1.00E+02	32.1	0.21
		32.36	1.00E+02		
		31.9	1.00E+02		
	NTC(DW)	32.33	0.00E+00		
		34.78	0.00E+00		
		31.69	0.00E+00		

	Sample	Cq	SQ	SQ AVG.	SQ STDEV.P	copy number
	SARS-CoV-2 2ng	17.42	2.47E+06	2.53E+06	0.0929	6.791E+06
		17.48	2.36E+06			
		17.26	2.75E+06			
	SARS-CoV-2 200pg	20.92	2.33E+05	2.33E+05	0.0327	6.254E+05
		20.88	2.39E+05			
		20.96	2.26E+05			
	SARS-CoV-2 20pg	24.53	2.04E+04	2.11E+04	0.0957	5.663E+04
		24.35	2.30E+04			
		24.57	1.98E+04			

dsDNA mass (3631bp)	Copy number
1ng	2.684.E+08
100pg	2.684.E+07
10pg	2.684.E+06
1pg	2.684.E+05
100fg	2.684.E+04
10fg	2.684.E+03
1fg	2.684.E+02

RNA	Copy number
2ng	6.8E+06
200pg	6.8E+05
20pg	6.8E+04
2pg	6.8E+03
200fg	6.8E+02
20fg	6.8E+01
2fg	6.8E+00

Figure S1. SARS-CoV-2 RNA copy number validation by RT-qPCR. SARS-CoV-2 genomic RNA copy number was validated by using ORF1ab target sequence fragment (RBC T&A-RTR 903) clone plasmid serial diluent RT-qPCR result (a) compare SARS-CoV-2 RNA serial diluent (2 ng–20 pg) using RT-qPCR results (b) that known plasmid copy number and SARS-CoV-2 RNA using RT-qPCR results (Ct value) converted to the equation which available estimating an average gene copy in SARS-CoV-2 genomic RNA (c).

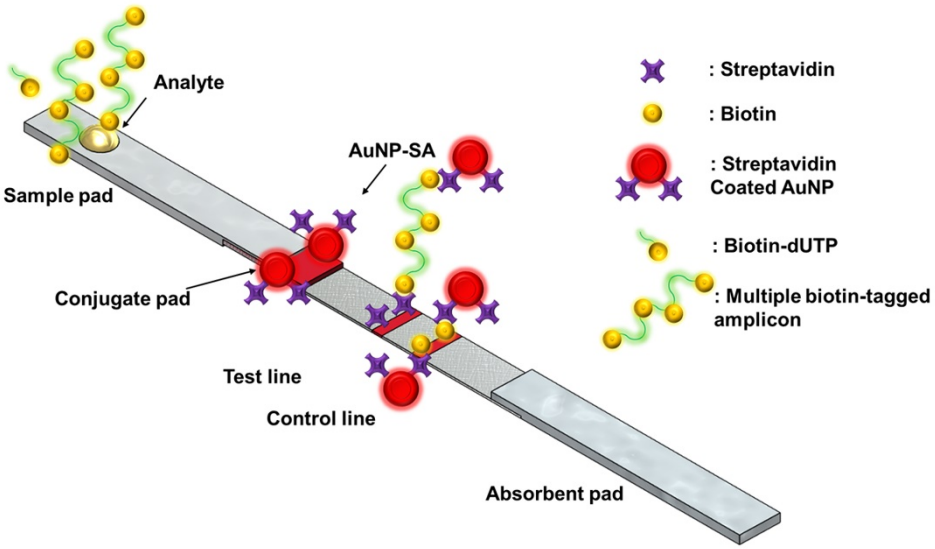


Figure S2. Schematic principle of LF assay readout.

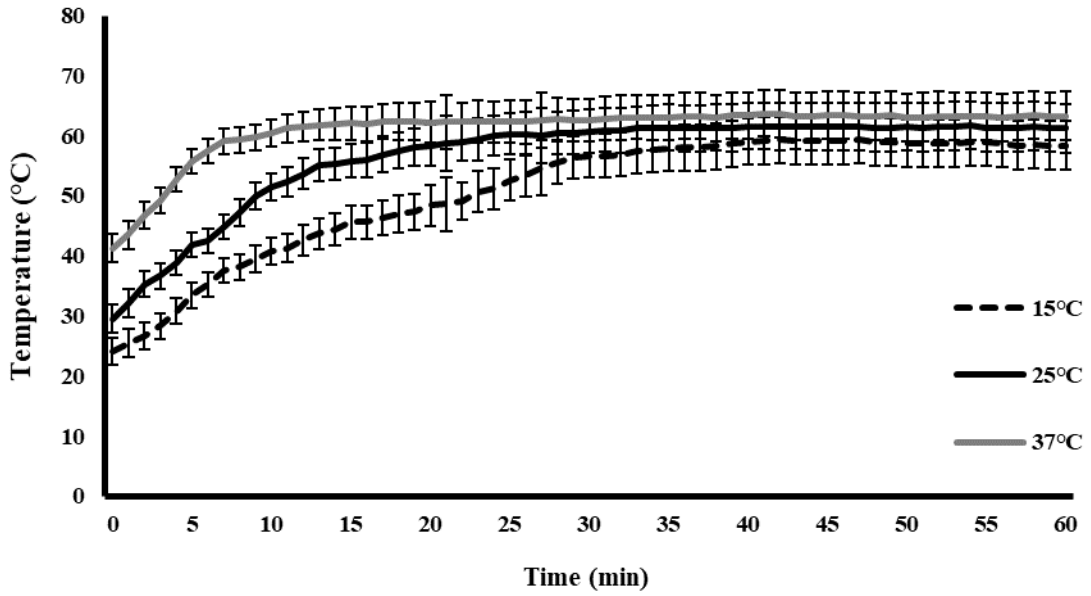


Figure S3. The temperature change of the hot-pack according to various ambient temperatures (15°C, 25°C, 37°C). The tests were repeated 5 times.

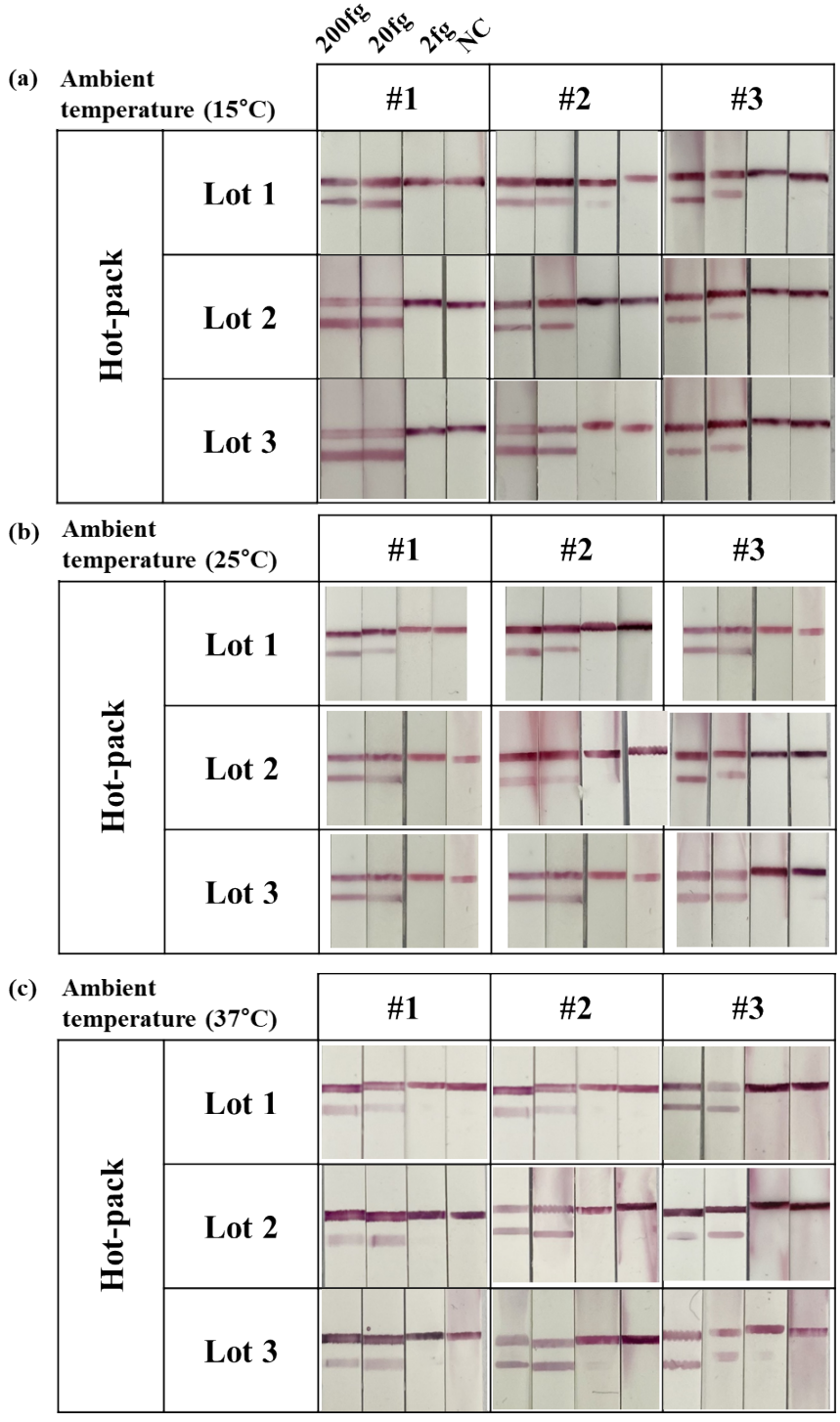


Figure S4. Device-to-device reproducibility with hot-pack (3 lots) and ambient temperature (15°C, 25°C, and 37°C). The tests were repeated 3 times.

Figure S5. COVID-19 clinical sample ORF1ab (RdRp) target RT-LAMP reactants LF assay results. Hot-pack applied RT-LAMP reaction assessed with COVID-19 clinical sample, which was read out by using LF assay. Left #1-44 positive sample detection and # 1, #27 weak positive sample showed negative results (red marked), Right #1-44 negative sample detection and #4, #9 were false positive.

Table S1. ORF1ab (RdRp) target RT-LAMP primer design

Primer set A		Ref.
F3	TTTATAGTGATGTAGAAAACCCTC	This study
B3	TAGTGAACCGCCACACAT	
FIP	CAAGTGAGGCCATAATTCTAAGCA-GGTTGGGATTATCCTAAATGTG	
BIP	TTCTTGCTCGCAAACATACAACG-ACTCAATACTTGAGCACACTC	
LF	ATAGAGCCATGCCTA	
LB	CTATAGAAACGGTGTGACAAGCTAC	

Primer set B		Ref.
F3	CCACTAGAGGAGCTACTGTA	This study
B3	TGACAAGCTACAACACGT	
FIP	AGGTGAGGGTTTTCTACATCACTAT-GGAACAAGCAAATTCTATGGT	
BIP	ATGGGTTGGGATTATCCTAAATGTG-TTGCGAGCAAGAACAAGT	
LF	GGCACAACATGTTAAAAACTG	
LB	CTAAGCATGTTAGGCATGGC	

Primer set C		Ref.
F3	GACCAATAGACAGTTTCATCAA	This study
B3	TCTAAGCATGTTAGGCATGG	
FIP	CACCATAGAATTTGCTTGTTCCAAT-AAATTATTGAAATCAATAGCCGC	
BIP	GTTGGCACAACATGTTAAAAACTGT-TCTATCACATTTAGGATAATCCCAA	
LF	CACTAGAGGAGCTACTGTAGTA	
LB	ATAAGGTGAGGGTTTTCTACATC	

Primer set D		Ref.
F3	CACCTTATGGGTTGGGAT	This study
B3	ATAGTGAACCGCCACACA	
FIP	GCAAGAACAAGTGAGGCCATA-ATCCTAAATGTGATAGAGCCA	
BIP	TCGCAAACATACAACGTGTTGT-TTTCACTCAATACTTGAGCAC	
LF	TGCCTAACATGCTTAGAAT	
LB	GCTAATCTATAGAAACGGTGTG	

Primer set E		Ref.
F3	CTATGGTGGTTGGCACAA	This study
B3	TTGAGCACACTCATTAGCT	

FIP	GCATGGCTCTATCACATTTAGGATA-ACTGTTTATAGTGATGTAGAAAACC	
BIP	ACATGCTTAGAATTATGGCCTCAC-TCTATAGAAACGGTGTGACAAG	
LF	CTCACCTTATGGGTTGGGAT	
LB	CGTTGTATGTTTGCGAGC	

Primer set F		**Ref.**
F3	CCTCAACTTGAACAGCCCT	**26**
B3	CGAAGAAGAACCTTGCGGT	
FIP	GCCTTCGAGTTCTGCTACCAGCTCATCAAACGTTCGGATGCT	
BIP	ACGGTCGTAGTGGTGAGACACTAAGCCACTGGTATTTCGCC	
LF	ACATGACCATGAGGTGCAGTTCG	
LB	TGGTGTCCTTGTCCCTCATGTG	

Primer set G		**Ref.**
F3	TGCTTCAGTCAGCTGATG	**27**
B3	TTAAATTGTCATCTTCGTCCTT	
FIP	CAGTACTAGTGCCTGTGCCGCACAATCGTTTTTAAACGGGT	
BIP	TCGTATACAGGGCTTTTGACATCTATCTTGGAAGCGACAACAA	
LF	CTGCACTTACACCGCAA	
LB	GTAGCTGGTTTTGCTAAATTCC	

ORF1ab target template cloning Primer set		
Forward primer	TGGTGGTGCATCGTGTTGTC	**This study**
Reverse primer	TCCACTACCTGGCGTGGTTT	

Seven sets of SARS-CoV-2 (Reference sequence ID: MN908947.3) of ORF1ab (RdRp) target RT-LAMP primers were designed using Primer Explorer V5 (http://primerexplorer.jp/e/), and RBC T&A-RTR 903 clones were selected from Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table S2. COVID-19 clinical sample validation results

Positive sample						
Sample number	**Allplex SARS-CoV-2 assay**					**ORF1ab (RdRp) target RT-LAMP reaction LF assay results**
	C(t)				**Results**	**Results**
	E gene	**RdRP/S gene**	**N gene**	**IC**		
Sample 1	**37.94**	**37.16**	**N/A**	**24.18**	**Weak Positive**	**Negative**
Sample 2	24.83	24.65	24.77	24.23	Positive	Positive
Sample 3	21.15	21.01	20.51	23.24	Positive	Positive
Sample 4	30.94	31.65	30.53	25.14	Positive	Positive
Sample 5	32.54	32.68	31.13	24.39	Positive	Positive
Sample 6	27.79	27.87	27.76	23.71	Positive	Positive
Sample 7	26.18	26.28	26.06	23.87	Positive	Positive
Sample 8	25.74	25.9	26.39	25.83	Positive	Positive
Sample 9	21.97	21.47	22.23	23.63	Positive	Positive
Sample 10	28.81	29.33	28.82	23.55	Positive	Positive
Sample 11	20.88	20.26	20.91	22.91	Positive	Positive
Sample 12	31.89	32.03	31.77	23.49	Positive	Positive
Sample 13	11.6	13.31	14.88	22.56	Positive	Positive
Sample 14	15.43	14.39	15.91	23.52	Positive	Positive
Sample 15	15.41	14.94	16.11	23.1	Positive	Positive
Sample 16	32.72	33.28	32.54	25.21	Positive	Positive
Sample 17	25.62	25.87	24.58	23.96	Positive	Positive
Sample 18	15.16	14.41	14.95	22.41	Positive	Positive
Sample 19	25.81	25.41	26.08	23.14	Positive	Positive
Sample 20	32.37	33.13	31.66	24.4	Positive	Positive
Sample 21	24	23.79	23.13	25.42	Positive	Positive
Sample 22	17.83	17.33	17.98	23.7	Positive	Positive
Sample 23	17.52	17.13	17.88	25.53	Positive	Positive
Sample 24	22.47	22.7	23.64	25.34	Positive	Positive
Sample 25	17.53	17.23	17.71	23.34	Positive	Positive
Sample 26	26.09	26.01	25.65	22.97	Positive	Positive
Sample 27	**37.81**	**37.27**	**37.62**	**25.49**	**Weak Positive**	**Negative**
Sample 28	27.9	27.86	27.83	24.87	Positive	Positive
Sample 29	27.54	27.45	26.99	23.3	Positive	Positive
Sample 30	28.65	29.23	28.74	23.45	Positive	Positive
Sample 31	26.39	26.26	26.24	23.58	Positive	Positive

Sample 32	19.73	19.28	19.91	23.96	Positive	Positive
Sample 33	23.34	22.95	22.79	25.03	Positive	Positive
Sample 34	32.46	33.13	31.15	23.79	Positive	Positive
Sample 35	16.05	15.48	13.14	25.02	Positive	Positive
Sample 36	26.93	26.86	24.89	25.1	Positive	Positive
Sample 37	25.33	25.47	24.28	24.63	Positive	Positive
Sample 38	24.31	23.86	24.86	23.36	Positive	Positive
Sample 39	33.11	33.86	32.6	23.66	Positive	Positive
Sample 40	22.65	22.43	23	23.66	Positive	Positive
Sample 41	27.73	28.23	27.9	23.45	Positive	Positive
Sample 42	24.54	24.62	24.15	23.02	Positive	Positive
Sample 43	28.98	29.9	28.34	23.46	Positive	Positive
Sample 44	32.54	32.71	32.12	24.01	Positive	Positive

Negative sample						
Sample number	**Allplex SARS-CoV-2 assay**					**ORF1ab (RdRp) target RT-LAMP reaction LF assay results**
	C(t)				**Results**	**Results**
	E gene	**RdRP/S gene**	**N gene**	**IC**		
Sample 1	N/A	N/A	N/A	24.18	Negative	Negative
Sample 2	N/A	N/A	N/A	22.91	Negative	Negative
Sample 3	N/A	N/A	N/A	23.49	Negative	Negative
Sample 4	**N/A**	**N/A**	**N/A**	**22.56**	**Negative**	**Positive**
Sample 5	N/A	N/A	N/A	23.52	Negative	Negative
Sample 6	N/A	N/A	N/A	23.45	Negative	Negative
Sample 7	N/A	N/A	N/A	23.58	Negative	Negative
Sample 8	N/A	N/A	N/A	23.96	Negative	Negative
Sample 9	**N/A**	**N/A**	**N/A**	**25.03**	**Negative**	**Positive**
Sample 10	N/A	N/A	N/A	23.79	Negative	Negative
Sample 11	N/A	N/A	N/A	25.02	Negative	Negative
Sample 12	N/A	N/A	N/A	23.49	Negative	Negative
Sample 13	N/A	N/A	N/A	23.34	Negative	Negative
Sample 14	N/A	N/A	N/A	25.03	Negative	Negative

Sample 15	N/A	N/A	N/A	23.79	Negative	Negative
Sample 16	N/A	N/A	N/A	25.02	Negative	Negative
Sample 17	N/A	N/A	N/A	23.3	Negative	Negative
Sample 18	N/A	N/A	N/A	23.45	Negative	Negative
Sample 19	N/A	N/A	N/A	23.14	Negative	Negative
Sample 20	N/A	N/A	N/A	24.63	Negative	Negative
Sample 21	N/A	N/A	N/A	23.36	Negative	Negative
Sample 22	N/A	N/A	N/A	22.66	Negative	Negative
Sample 23	N/A	N/A	N/A	23.66	Negative	Negative
Sample 24	N/A	N/A	N/A	25.34	Negative	Negative
Sample 25	N/A	N/A	N/A	23.34	Negative	Negative
Sample 26	N/A	N/A	N/A	25.03	Negative	Negative
Sample 27	N/A	N/A	N/A	23.79	Negative	Negative
Sample 28	N/A	N/A	N/A	25.02	Negative	Negative
Sample 29	N/A	N/A	N/A	23.3	Negative	Negative
Sample 30	N/A	N/A	N/A	23.45	Negative	Negative
Sample 31	N/A	N/A	N/A	23.58	Negative	Negative
Sample 32	N/A	N/A	N/A	23.96	Negative	Negative
Sample 33	N/A	N/A	N/A	25.03	Negative	Negative
Sample 34	N/A	N/A	N/A	23.79	Negative	Negative
Sample 35	N/A	N/A	N/A	25.02	Negative	Negative
Sample 36	N/A	N/A	N/A	25.1	Negative	Negative
Sample 37	N/A	N/A	N/A	24.63	Negative	Negative
Sample 38	N/A	N/A	N/A	23.36	Negative	Negative
Sample 39	N/A	N/A	N/A	23.45	Negative	Negative
Sample 40	N/A	N/A	N/A	23.58	Negative	Negative
Sample 41	N/A	N/A	N/A	23.96	Negative	Negative
Sample 42	N/A	N/A	N/A	25.03	Negative	Negative
Sample 43	N/A	N/A	N/A	23.79	Negative	Negative
Sample 44	N/A	N/A	N/A	23.96	Negative	Negative

Table S3. Calculation of the cost of the pre-integrated RT-LAMP system.

Reagents	**Cost per device ($)**
Primers	0.03
Bst Polymerase	0.06
Reverse Transcriptase	0.07
dNTPs	0.03
Biotin-dUTP	0.015
RT-LAMP buffer (Tris-HCl, KCl..)	0.0002
Backing card, NC membrane, sample pads	0.06
Streptavidin	0.1
Biotin-BSA	0.05
Reagents (AuNPs, buffers, and chemicals)	0.05
Packaging (Tubes, housing, desiccant)	0.03
Total cost	**0.4952** (Calculated from retail cost in south Korea)