Supporting Information

Dual-Priming Isothermal Amplification (DAMP) for Highly Sensitive and Specific Molecular Detection with Ultralow Nonspecific Signals

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Table S1. List of sequence information of the primers and targets.

Item	Sequence (5'-3')	Description
For the amplification of HIV-1 p24 gene sequence		
The 300-bp HIV-1 p24 gene sequence inserted into the pUCIDT (Amp) plasmid	CCAGAAGTAATACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGA TTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATGCAAATGT TAAAAGAAACCATCAATGAGGAAGCTGCAGAATGGGATAGATTGCATCCCGTG CAGGCAGGGCCTGTTGCACCAGGCCAGATAAGAGATCCAAGGGGAAGTGACAT AGCAGGAACTACCAGTACCCTTCAGGAACAAATAGGATGGATGACAAGTAATC CACCTATCCCAGTAGGAGAAATCTATAAAAGATGG	
FO primer	ATTATCAGAAGGAGCCACC	
RO primer	GGGATAGGTGGATTACTTGT	
FC primer	TCTGCAGCTTCCTCATTGATGG	
RC primer	TTGCACCAGGCCAGATAAGA	
FI primer	TCTGCAGCTTCCTCATTGATGG**TTTT**ATCAAGCAGCCATGCAAAT	Inner primers using target sites with 30 nt distance
RI primer	TTGCACCAGGCCAGATAAGA**TTTT**AGTTCCTGCTATGTCACTT	Inner primers using target sites with 30 nt distance
FI primer	TCTGCAGCTTCCTCATTGATGG**TTTT**ACCATGCTAAACACAGTGG	Inner primers using target sites with 55 nt distance
RI primer	TTGCACCAGGCCAGATAAGA**TTTT**CCTATTTGTTCCTGAAGGGTAC	Inner primers using target sites with 56 nt distance
FI primer	TCTGCAGCTTCCTCATTGATGG**TTTT**GCCATGCAAATGTTAAAAGAAA	Inner primers using target sites with 22 nt distance
RI primer	TCTGCAGCTTCCTCATTGATGG**TTTT**GTCACTTCCCCTTGGATC	Inner primers using target sites with 22 nt distance
FI primer	ATCAAGCAGCCATGCAAAT	Only "pairing-priming" DAMP
RI primer	AGTTCCTGCTATGTCACTT	Only "pairing-priming" DAMP

F3 primer	ATTATCAGAAGGAGCCACC	LAMP using the same amplification region
B3 primer	GGGATAGGTGGATTACTTGT	LAMP using the same amplification region
FIP primer	TCTGCAGCTTCCTCATTGATGG**TTTT**ACCATGCTAAACACAGTGG	LAMP using the same amplification region
BIP primer	TTGCACCAGGCCAGATAAGA**TTTT**CCTATTTGTTCCTGAAGGGTAC	LAMP using the same amplification region
LoopF primer	ATTTGCATGGCTGCTTGATGTC	LAMP using the same amplification region
LoopB primer	GAAGTGACATAGCAGGAACTACCA	LAMP using the same amplification region
Swarm primer 1	TCTGCAGCTTCCTCATTGATGG	Swarm primer added to the LAMP above
Swarm primer 2	TTGCACCAGGCCAGATAAGA	Swarm primer added to the LAMP above
F primer	ATTATCAGAAGGAGCCACC	PCR
B primer	CATCCTATTTGTTCCTGAAGG	PCR
For the amplification of *E. coli* B *mal*B gene sequence		
The 300-bp *E. coli* B *mal*B gene sequence inserted into the pUCIDT (Amp) plasmid	GCCAGGGGGTGGAGGATTTAAGCCATCTCCTGATGACGCATAGTCAGCCCATC ATGAATGTTGCTGTCGATGACAGGTTGTTACAAAGGGAGAAGGGCATGGCGAG CGTACAGCTGCAAAATGTAACGAAAGCCTGGGGCGAGGTCGTGGTATCGAAAG ATATCAATCTCGATATCCATGAAGGTGAATTCGTGGTGTTTGTCGGACCGTCT GGCTGCGGTAAATCGACTTTACTGCGCATGATTGCCGGGCTTGAGACGATCAC CAGCGGCGACCTGTTCATCGGTGAGAAACGGATGA	
FO primer	GCTGTCGATGACAGGTTGTT	DAMP
RO primer	ATTTACCGCAGCCAGACG	DAMP
FI primer	TTTTGCAGCTGTACGCTCG**TTTT**CAAAGGGAGAAGGGCATGG	DAMP

RI primer	ATCAATCTCGATATCCATGAAGGTG**TTTT**TCCGACAAACACCACGAATT	DAMP
FC primer	TTTTGCAGCTGTACGCTCG	DAMP
RC primer	ATCAATCTCGATATCCATGAAGGTG	DAMP
F3 primer	GCCATCTCCTGATGACGC	The reported LAMP[1]
B3 primer	ATTTACCGCAGCCAGACG	The reported LAMP[1]
FIP primer	CATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGCT	The reported LAMP[1]
BIP primer	CTGGGGCGAGGTCGTGGTATTCCGACAAACACCACGAATT	The reported LAMP[1]
LoopF primer	CTTTGTAACAACCTGTCATCGACA	The reported LAMP[1]
LoopB primer	ATCAATCTCGATATCCATGAAGGTG	The reported LAMP[1]
F primer	GCCATCTCCTGATGACGC	PCR
B primer	ATTTACCGCAGCCAGACG	PCR

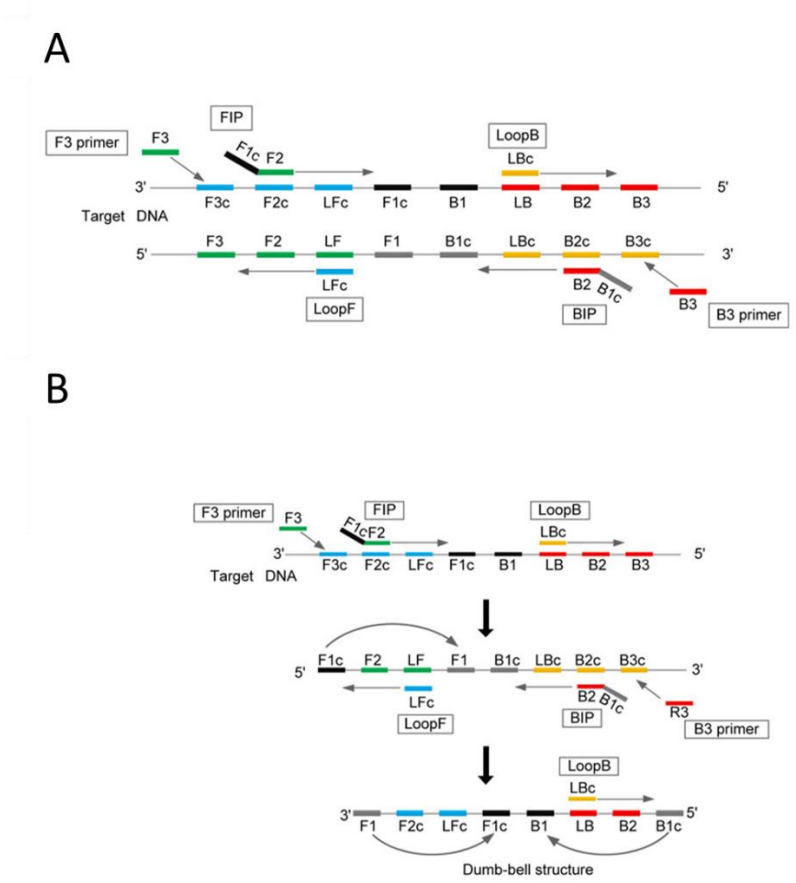

Figure S1. Principle of loop-mediated isothermal amplification (LAMP) with two loop primers (six-primer LAMP) method.[2,3] (A) Primer design of the six-primer LAMP reaction. (B) Dumb-bell structure producing step of six-primer LAMP.

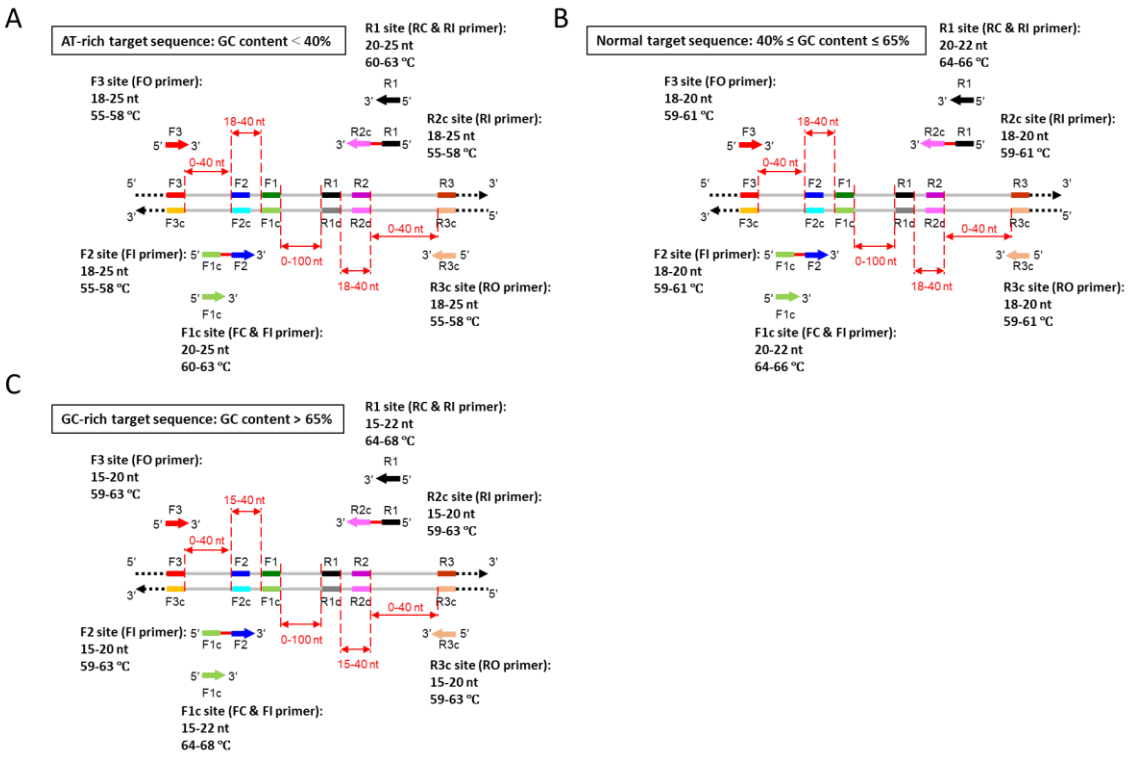

Figure S2. Length and melting temperature (T_m) for each target site used for DAMP primer design when the target sequence is AT-rich (A), Normal (B), and GC-rich (C).

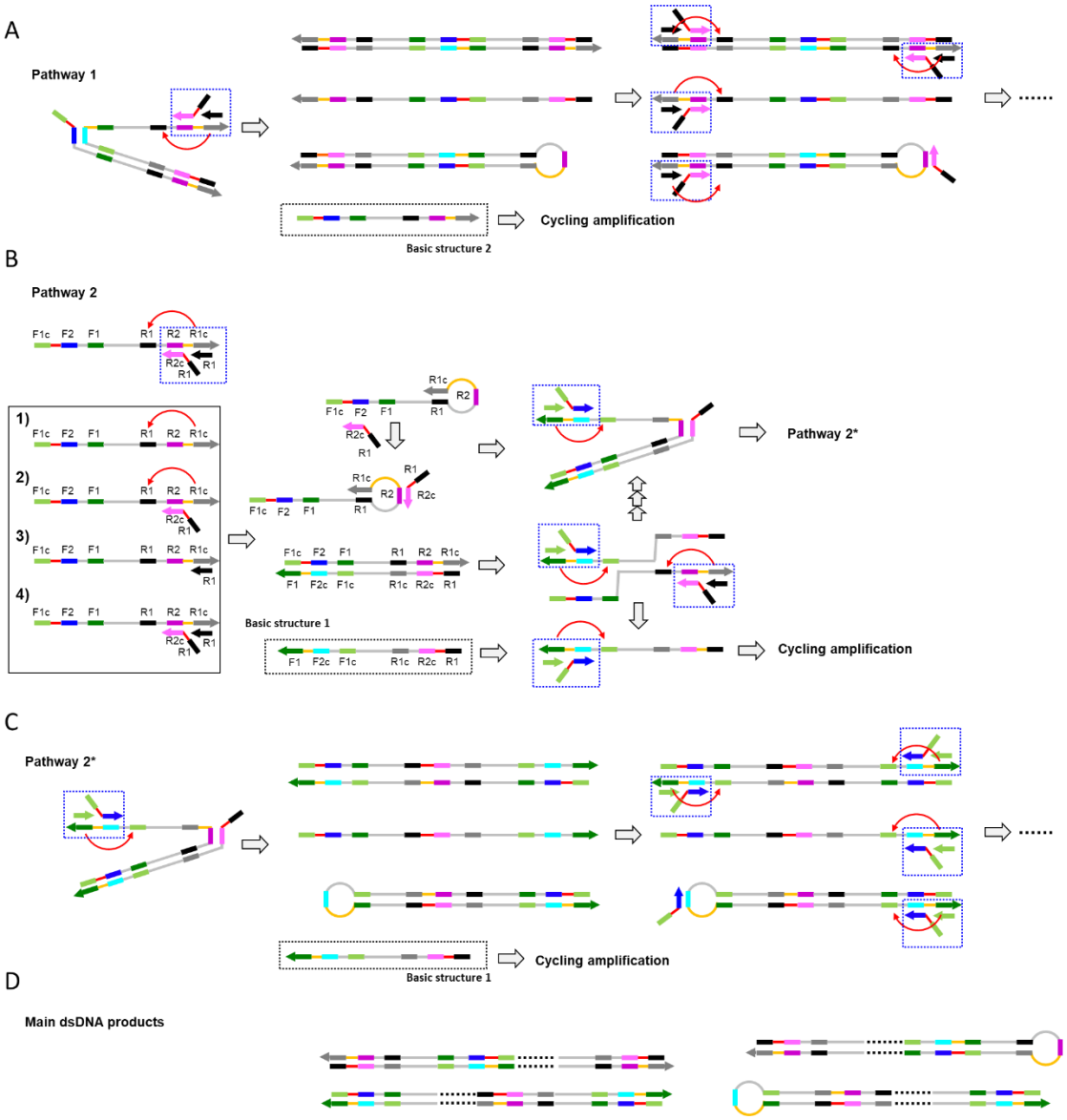

Figure S3. Continued cycling amplification step for DAMP reaction includes: (A) Pathway 1, (B) Pathway 2, and (C) the complementary Pathway 2 (Pathway 2*). (D) The main dsDNA products in DAMP reactions.

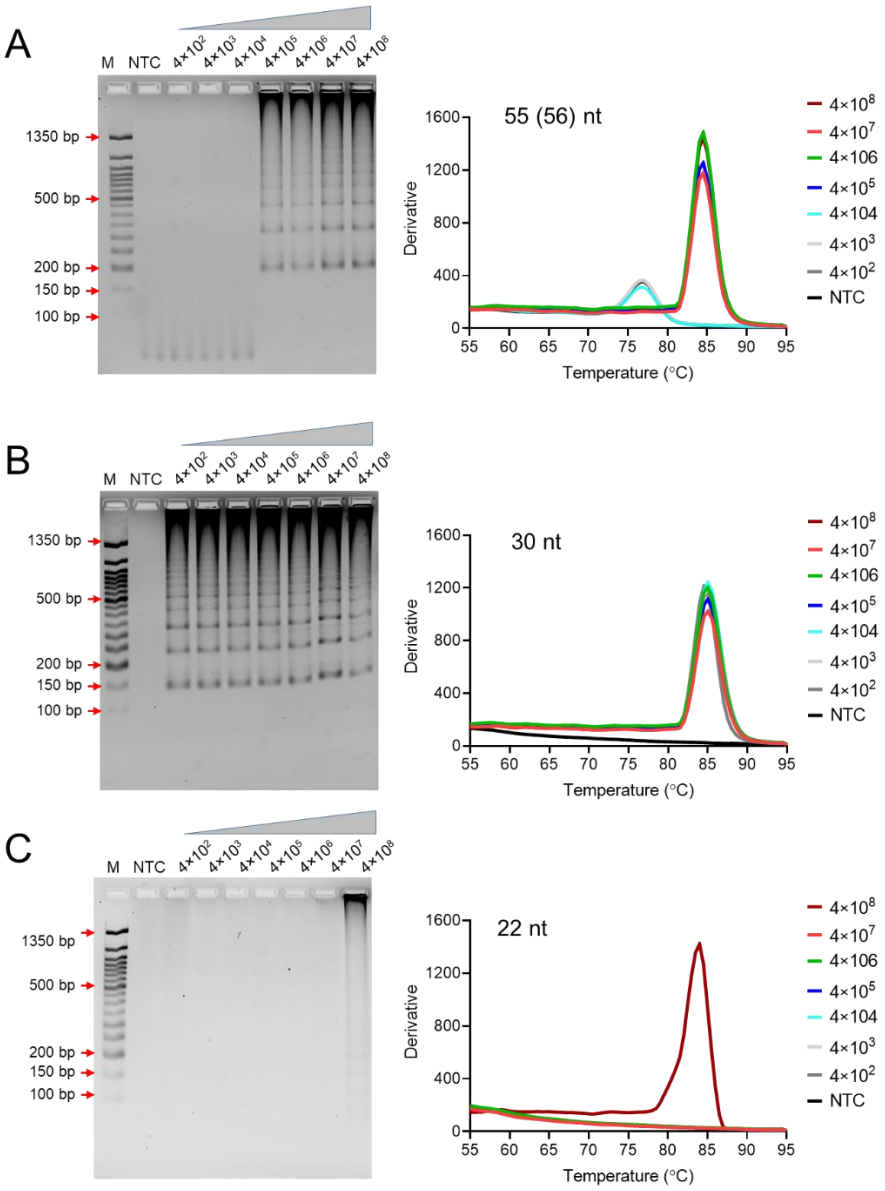

Figure S4. Agarose gel electrophoresis analysis and the melting curve analysis of the products of DAMP assays with (A) 55/56 nt, (B) 30 nt, and (C) 22 nt distance of the two target sites for each inner primer design to detect various copies of targets (from 4×10^8 to 4×10^2 copies). M, 50 bp DNA ladder marker. NTC, non-template control.

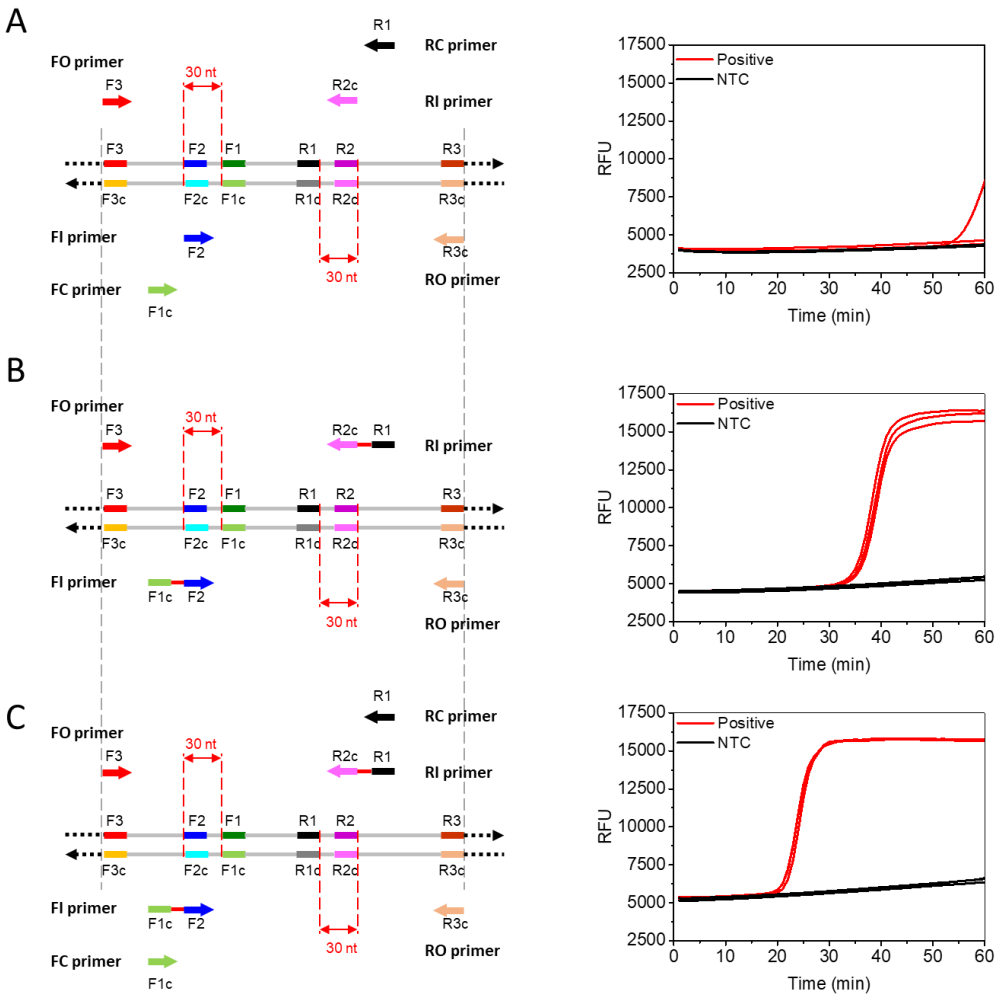

Figure S5. Evaluate the influence of various primer designs on DAMP efficiency. (A) Primer design with single-site inner primers (Left) and its real-time fluorescence isothermal amplification curves (Right). (B) Primers design without including FC and RC primers (Left) and the real-time fluorescence isothermal amplification curves (Right). (C) Primer design of typical DAMP (Left) and its real-time fluorescence DAMP curves (Right). The 300-bp HIV-1 p24 gene cDNA sequence was used as the target sequence which was inserted into a plasmid. The vertical gray dash line denotes the same amplification region. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

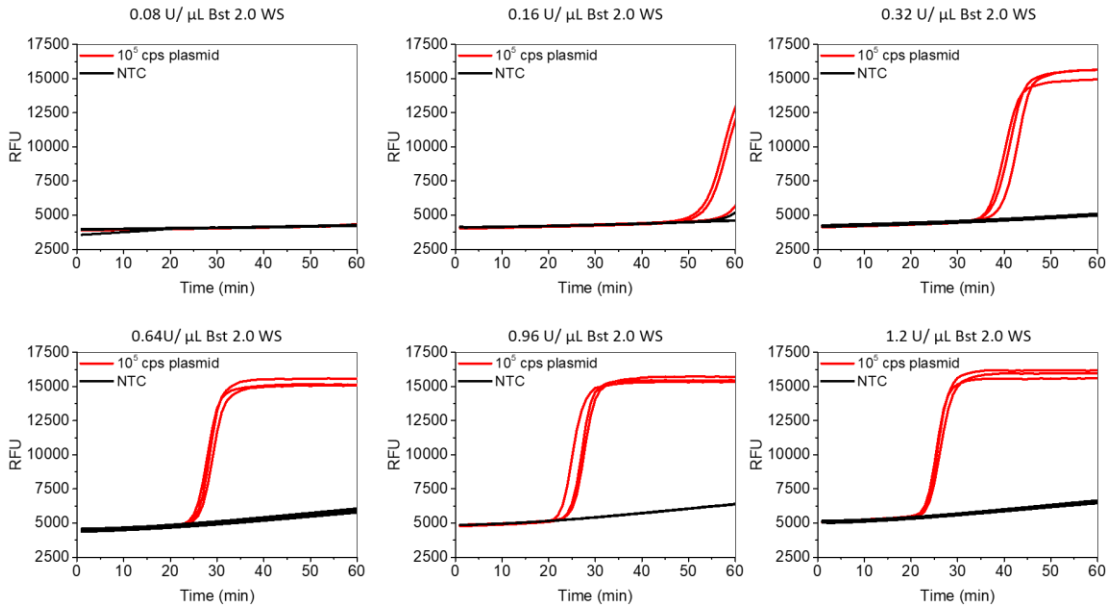

Figure S6. Real-time fluorescence signals of DAMP assay using various amounts of Bst 2.0 WarmStart DNA polymerase (Bst 2.0 WS). The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

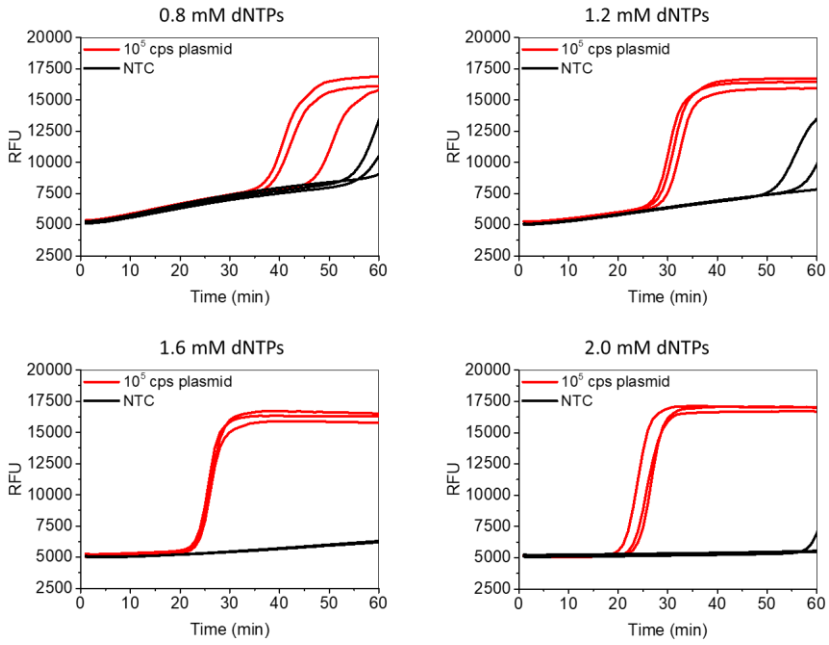

Figure S7. Real-time fluorescence signals of DAMP assay using various concentrations of dNTPs. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

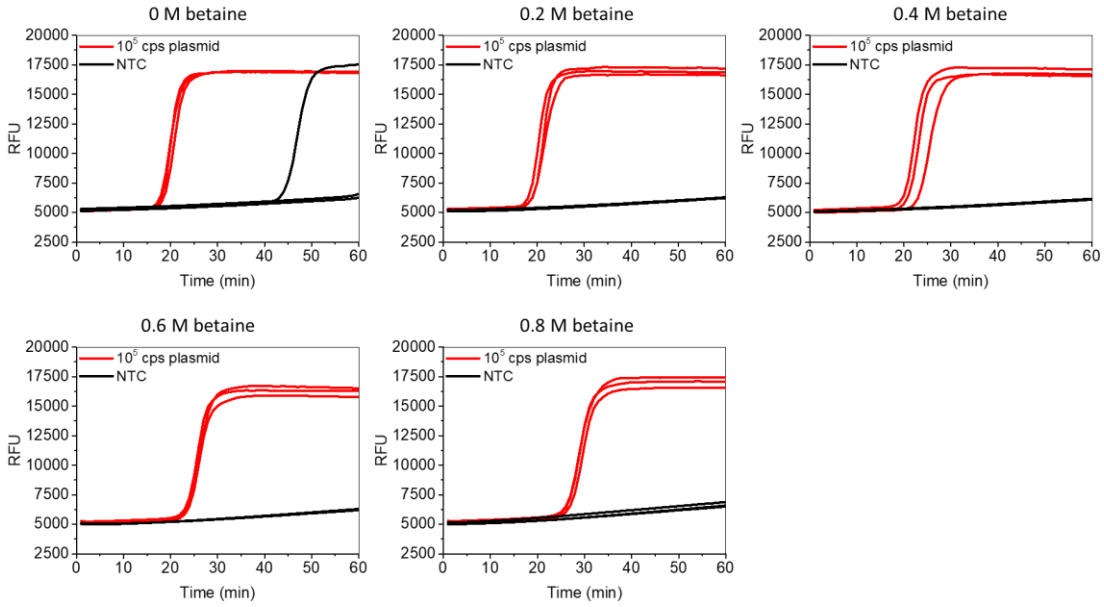

Figure S8. Real-time fluorescence signals of DAMP assay using various concentrations of betaine. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

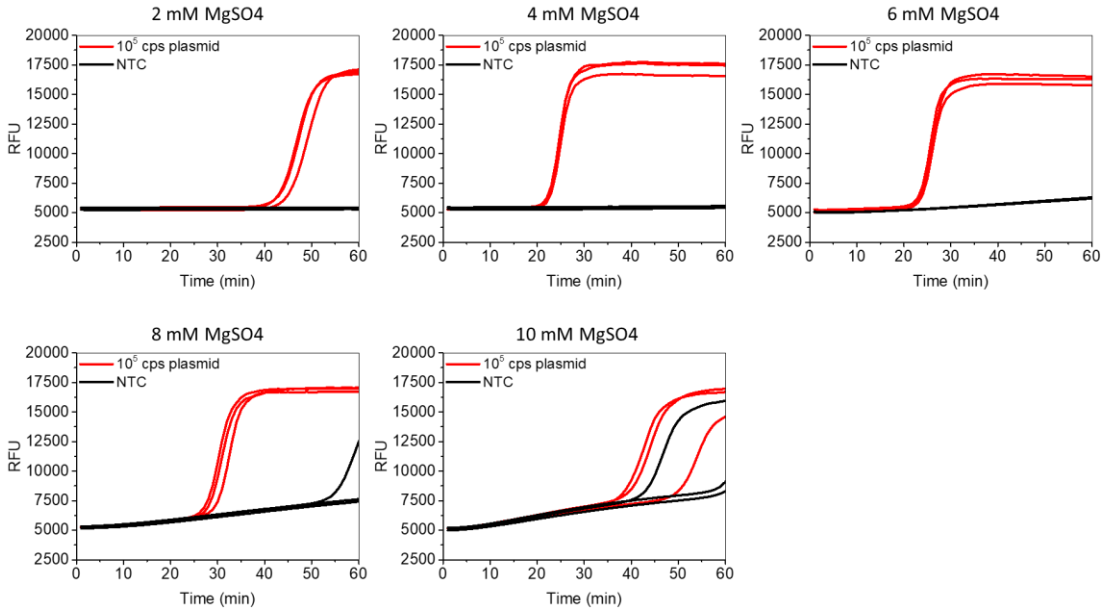

Figure S9. Real-time fluorescence signals of DAMP assay using various concentrations of $MgSO_4$. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

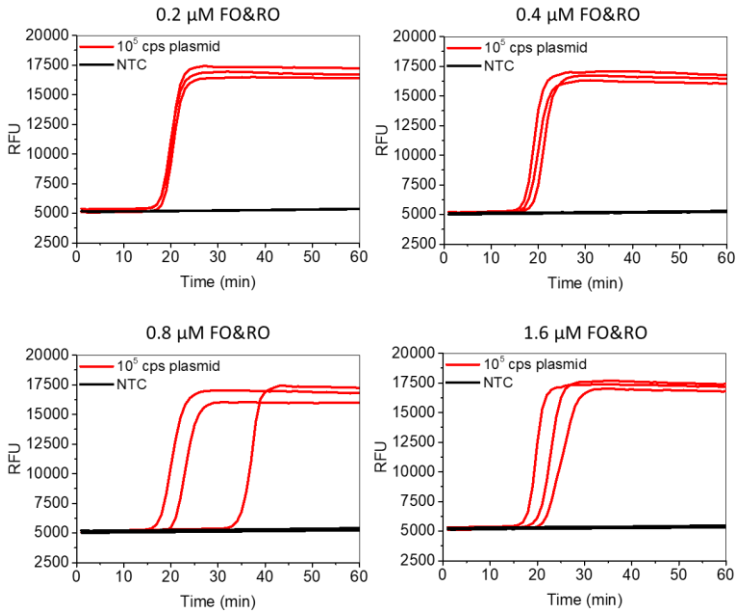

Figure S10. Real-time fluorescence signals of DAMP assay using various concentrations of outer primers FO and RO. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

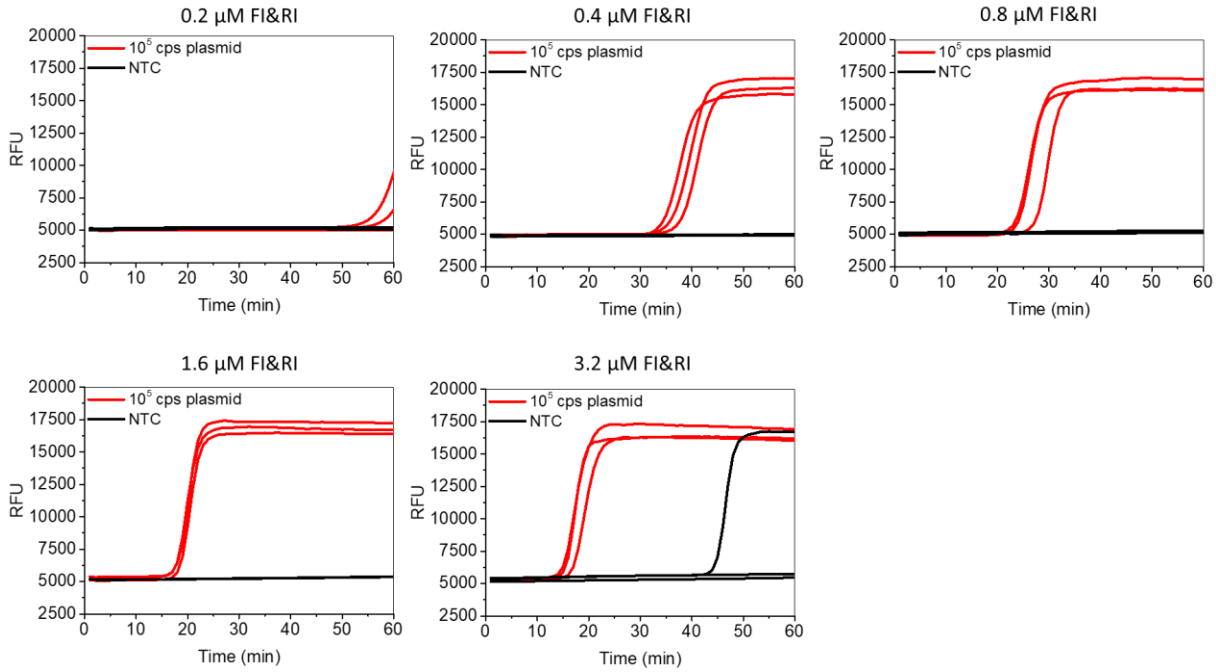

Figure S11. Real-time fluorescence signals of DAMP assay using various concentrations of inner primers FI and RI. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

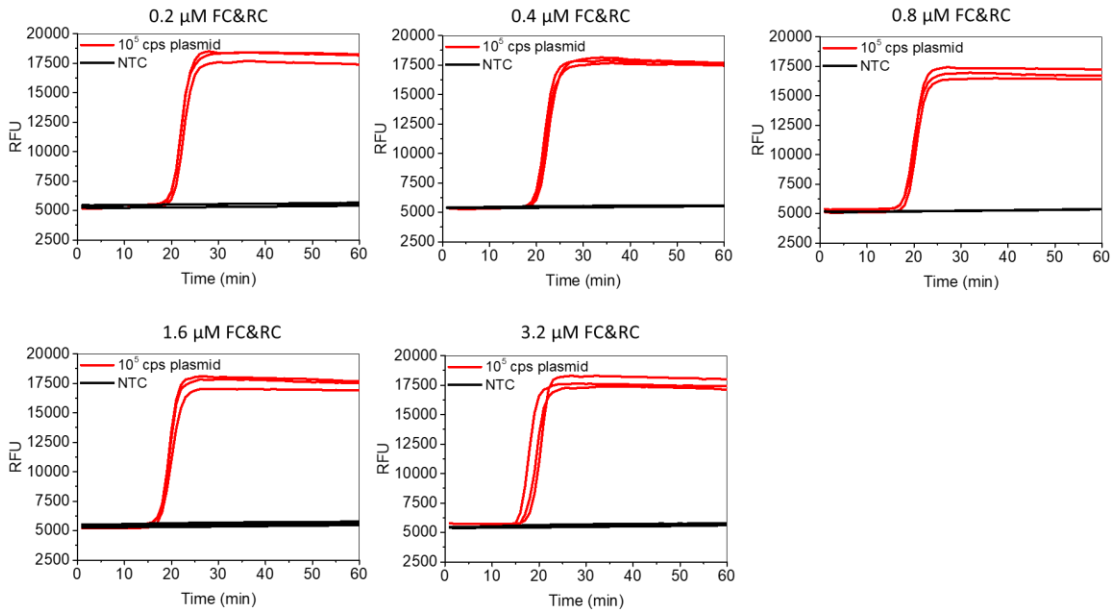

Figure S12. Real-time fluorescence signals of DAMP assay using various concentrations of pairing-competition primers FC and RC. The 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

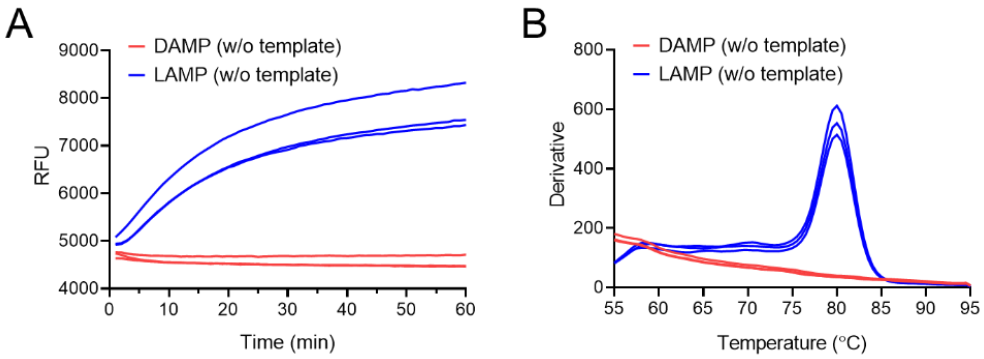

Figure S13. (A) Real-time fluorescence curves and (B) melting curve analysis of the DAMP and LAMP products of non-template control (NTC). Three replicates ran for each reaction or test.

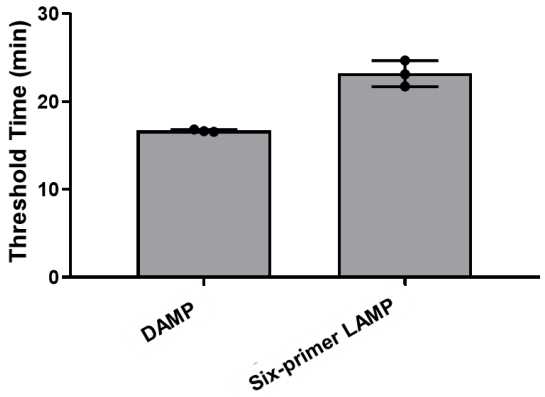

Figure S14. Threshold time comparison of 10^5 copies of PV DNA templates detected by the DAMP and the six-primer LAMP. The 300-bp HIV-1 p24 gene cDNA sequence was the target sequence. The positive was the reaction with 10^5 copies templates. Error bars represent the standard deviations at three replicates (n = 3).

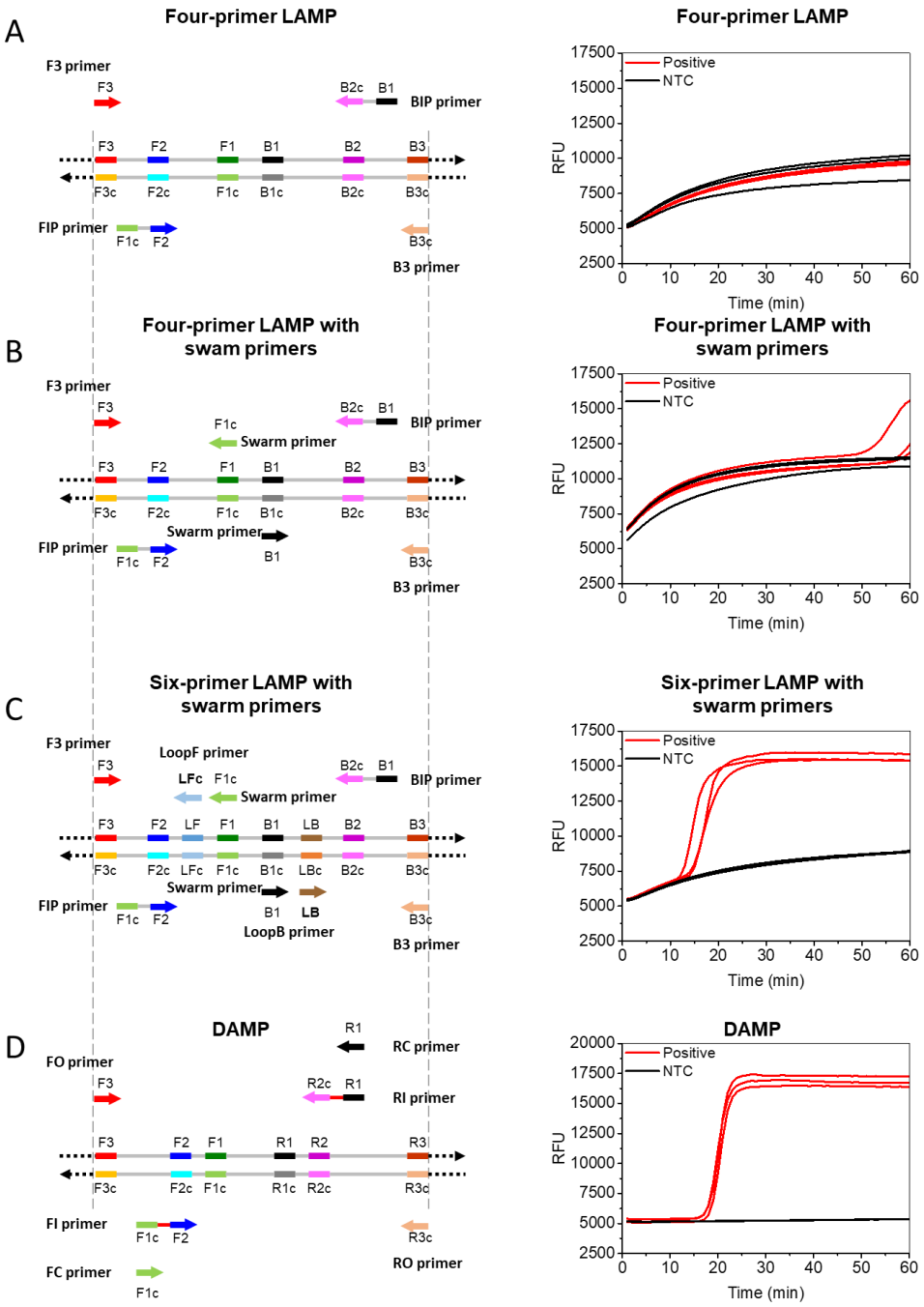

Figure S15. Comparison of DAMP with different LAMP variants. (A) Four-primer LAMP primer design (Left) and its real-time fluorescence signals (Right). (B) Four-primer LAMP with swarm primers (Left) and its real-time fluorescence signals (Right). (C) Six-primer LAMP with swarm primers (Left) and its real-time fluorescence signals (Right). (D) DAMP (Left) and its real-time fluorescence signals (Right). 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. The vertical gray dash line denotes the same amplification region. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

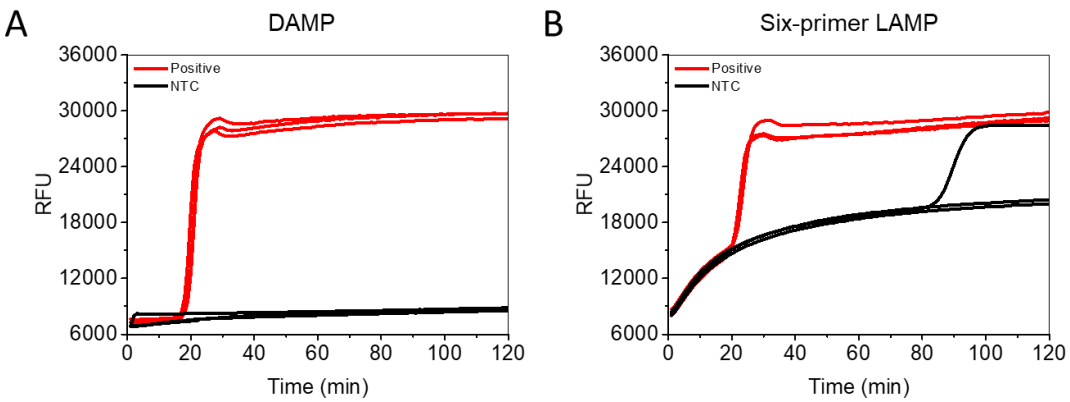

Figure S16. Real-time fluorescence signals of (A) DAMP and (B) six-primer LAMP to amplify the same target sequence (HIV DNA) in two-hour incubation. 300-bp HIV-1 p24 gene cDNA sequence inserted into a plasmid was the target sequence. Positive, three replicated reactions with 10^5 copies templates. NTC, three replicated non-template control reactions.

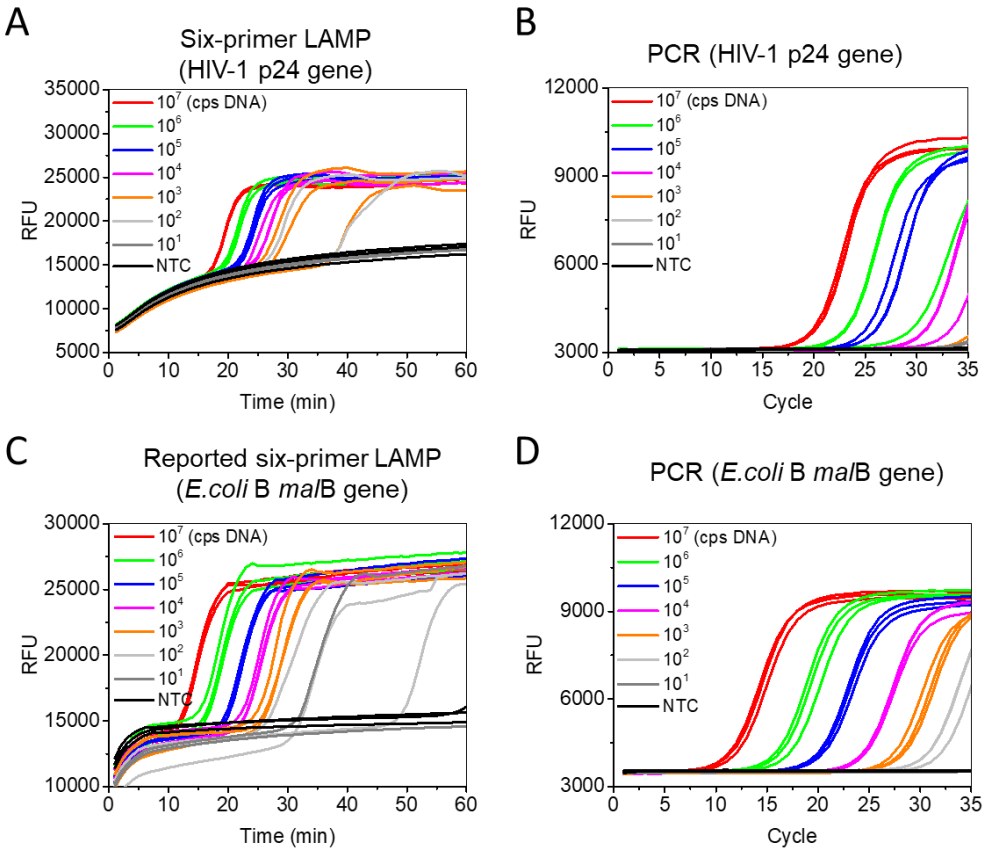

Figure S17. (A) Sensitivity of DAMP assay for the detection of HIV-1 p24 gene cDNA sequence. (B) Sensitivity of PCR assay for the detection of HIV-1 p24 gene cDNA sequence. (C) Sensitivity of DAMP assay for the detection of *E. coli* B *mal*B gene sequences. (D) Sensitivity of PCR assay for the detection of *E. coli* B *mal*B gene sequences. Error bars represent the standard deviations at three replicates (n = 3). Cps, copy number. Three replicates were set to amplify each cps of targets. NTC, three replicated non-template control reactions.

Copies of plasmid templates with p24 gene sequences	No. of positive tests / No. of total tests	
	DAMP	LAMP
10^4	6/6	6/6
10^3	6/6	6/6
10^2	5/6	3/6
80	2/6	2/6
60	1/6	0/6
40	0/6	1/6
20	1/6	0/6
10	0/6	0/6
5	0/6	0/6
2	0/6	0/6

Figure S18. Summary of the ratio of positive tests for replicated DAMP and LAMP assays to detect the templates of plasmids containing p24 gene sequences. The 95% detection limit of the real-time DAMP and LAMP assays are approximately 210.73 and 567.44 copies of plasmids using the probit analysis, respectively.

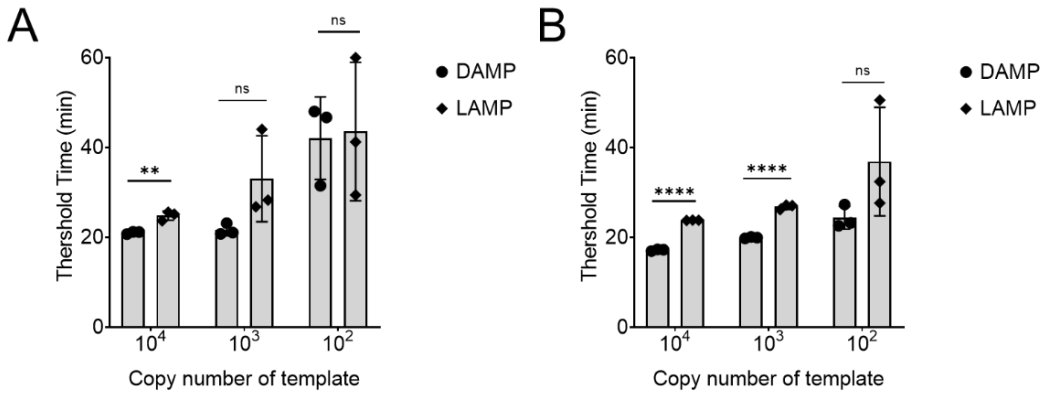

Figure S19. Comparison of threshold time for DAMP and LAMP using the templates with copies number at 10^4, 10^3, and 10^2. (A) For the detection of p24 gene sequence. (B) For the detection of *mal*B gene sequence. Three replicates ran for each reaction or test. Error bars represent the standard deviations at three replicates (n = 3). Unpaired two-tailed t test was used to analyse the difference. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

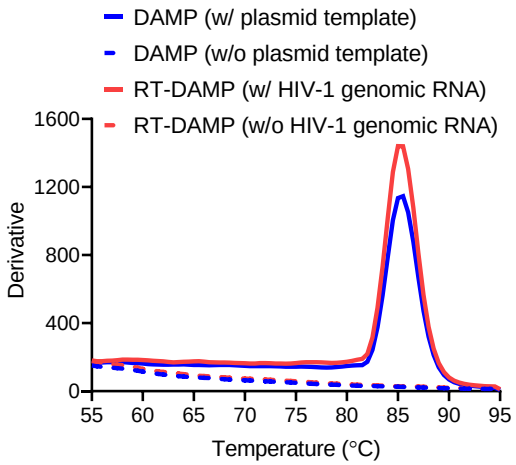

Figure S20. Melting curves of the DAMP product of plasmids containing HIV-1 p24 gene (100 copies) and the RT-DAMP product of HIV-1 genomic RNA (350 copies) extracted from HIV virus in human plasma.

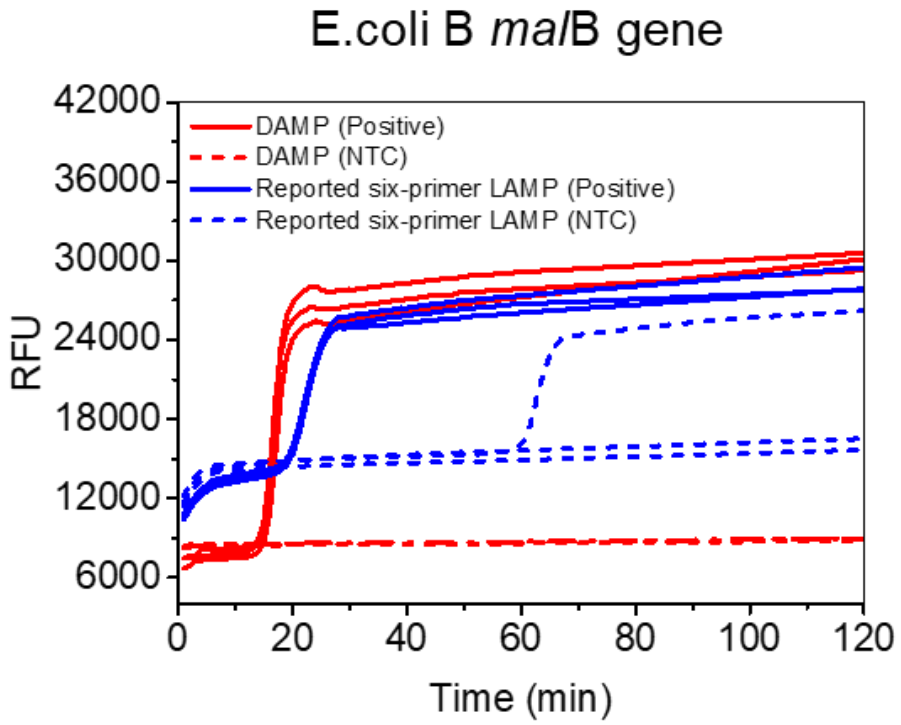

Figure S21. Real-time fluorescence signals of DAMP and the reported six-primer LAMP for the amplification of *E. coli* B *mal*B gene sequences within two-hour incubation. Positive, three replicated reactions with 10^5 copies templates are shown.

References

(1) Hill, J.; Beriwal, S.; Chandra, I.; Paul, V. K.; Kapil, A.; Singh, T.; Wadowsky, R. M.; Singh, V.; Goyal, A.; Jahnukainen, T. *J. Clin. Microbiol.* **2008**, *46*, 2800-2804.
(2) Ding, X.; Nie, K.; Shi, L.; Zhang, Y.; Guan, L.; Zhang, D.; Qi, S.; Ma, X. *J. Clin. Microbiol.* **2014**, *52*, 1862-1870.
(3) Nagamine, K.; Hase, T.; Notomi, T. *Mol. Cell. Probes* **2002**, *16*, 223-229.