Supporting Information for

Ultrasensitive version of nucleic acid sequence-based amplification (NASBA) utilizing nicking and extension chain reaction system

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Table S1. Oligonucleotide sequences employed in this study

Oligonucleotide	Sequence (5'→3')[a]
NASBA primer 1	**AAT TCT AAT ACG ACT CAC TAT AGG G**AC TAA TGG TGC TAG TGA C
NASBA primer 2	GTG ATT CAA CAA TGA CCA A
NESBA primer 1 (NP1)	**AAA AAA AGG ATC GGG GAA TTC TAA TAC GAC TCA CTA TAG GG**A CTA ATG GTG CTA GTG AC
NESBA primer 2 (NP2)	**AAA AAA AGG ATC GGG G**GT GAT TCA ACA ATG ACC AA
Molecular beacon (MB)[b]	FAM[1]-CTT GGA TTT GAT CTT AAT TCC AAG-DABCYL[2]

(a) The underlined letters indicate the T7 RNA promoter sequence and the bold letters indicate the extended sequence of the primer. Green color represents the nicking site and blue color in MB represents the sequence binding to RNA amplicon.

(b) Abbreviations: (1) FAM: carboxyfluorescein, (2) DABCYL: 4-([4-(Dimethylamino) phenyl]azo)benzoic acid.

Table S2. Reaction conditions of different types of NE

NE	Optimal temperature (℃)	Reaction buffer
Nt.AlwI	**37**	**NEbuffer™ 2.1[(a)]**
Nt.BsmAI	**37**	**CutSmart™ Buffer[(b)]**
Nt.BstNBI	**55**	**NEbuffer™ 3.1[(c)]**
Nb.BsmI	**65**	**NEbuffer™ 3.1**

(a) NEbuffer™ 2.1 (1X): 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, and 100 μg/ml BSA

(b) CutSmart™ Buffer (1X): 20 mM Tris-acetate, pH 7.9, 50 mM Potassium acetate, 10 mM Magnesium acetate, and 100 μg/ml Recombinant Albumin

(c) NEbuffer™ 3.1 (1X): 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, and 100 μg/ml Recombinant Albumin

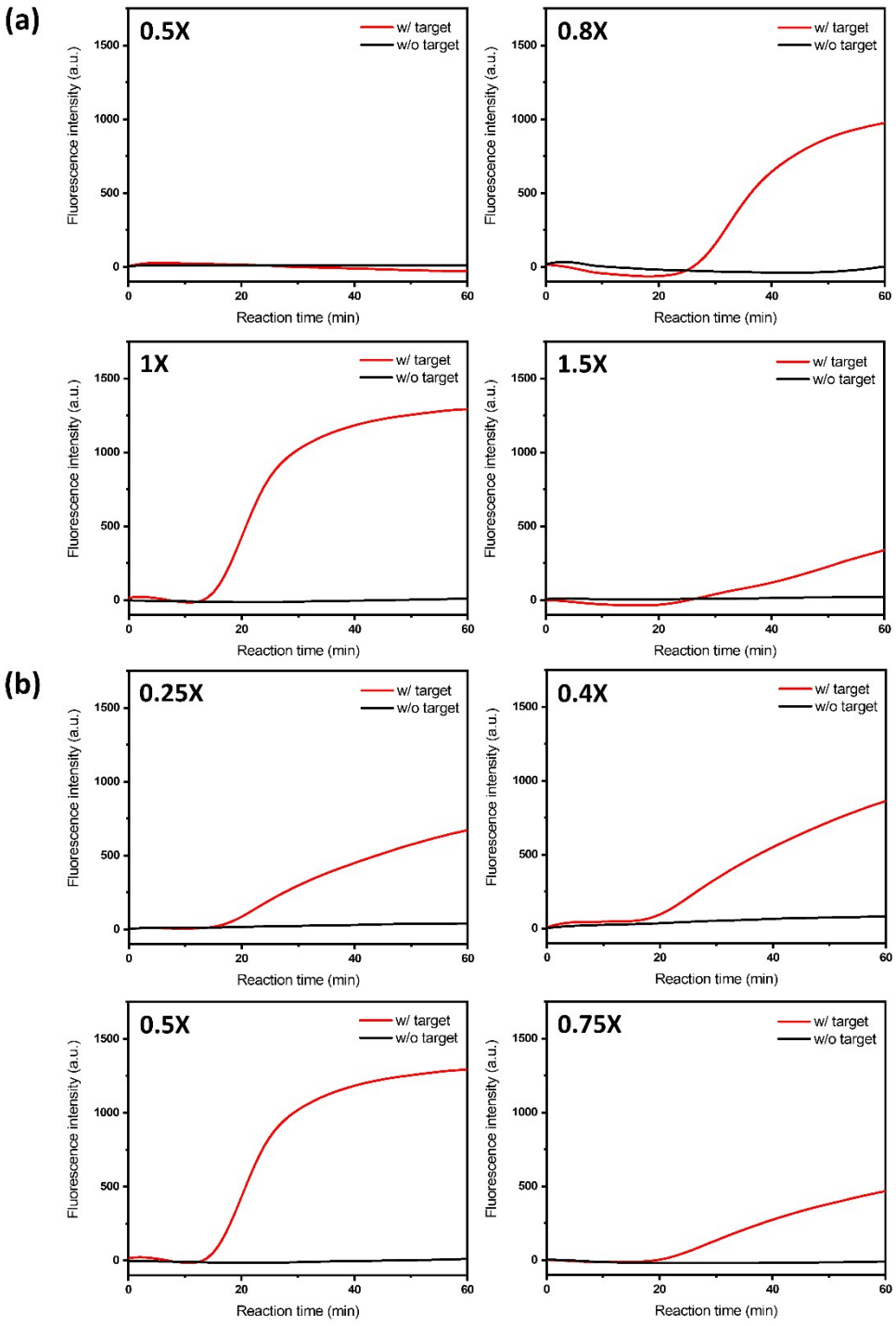

Fig. S1. Optimization of NASBA reaction buffer and NEBuffer™ 2.1. Time-dependent fluorescent signals produced from MB during the NESBA reaction containing (a) NASBA reaction buffer and (b) NEBuffer™ 2.1 at varying concentrations in the presence (Red) and

absence of target gRNA (Black). The final concentrations of NASBA enzyme cocktail, NE, and a NESBA primer set are 1×, 0.25 U/μL, and 0.7 μM each, respectively.

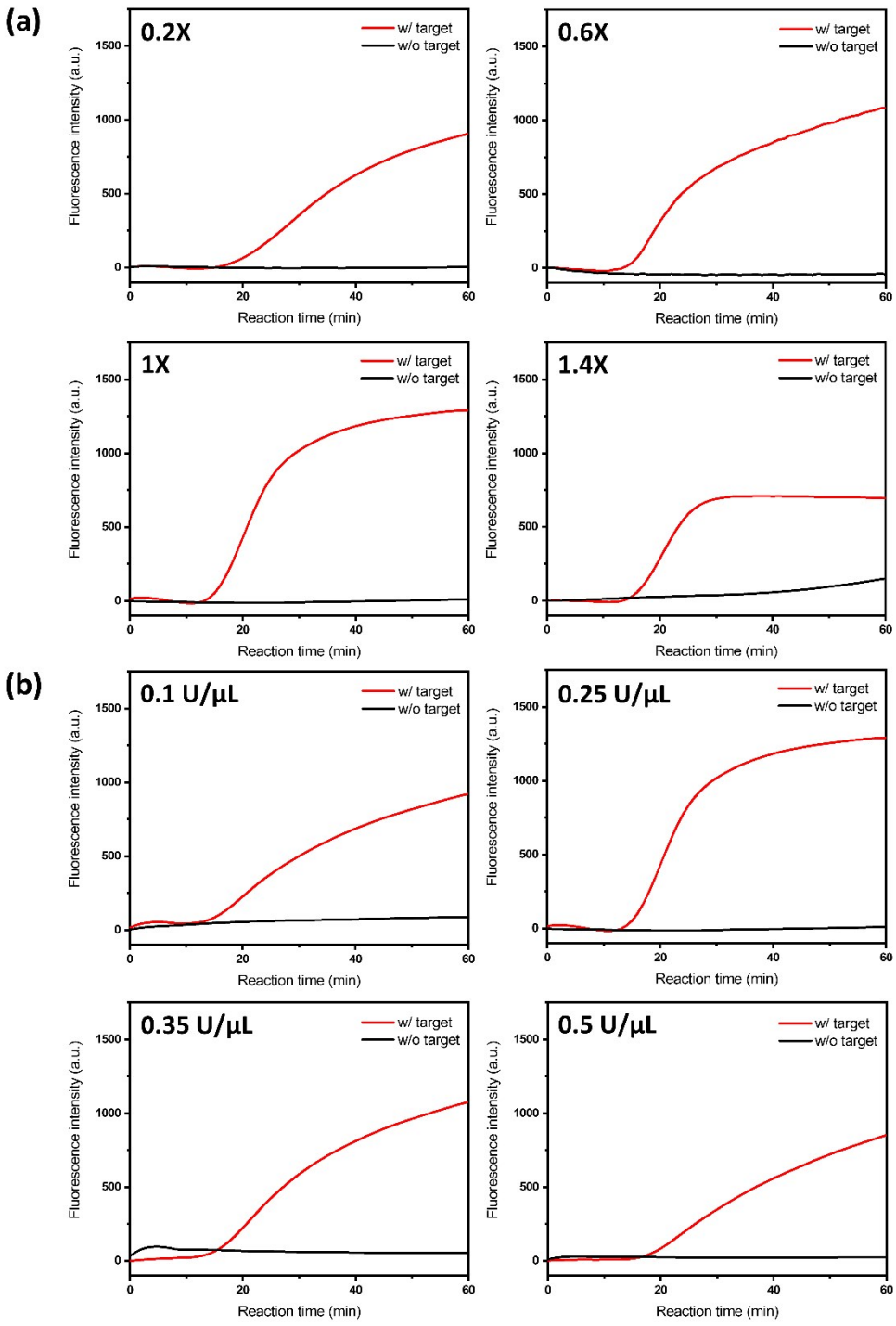

Fig. S2. Optimization of the NASBA enzyme cocktail and NE concentrations. Time-dependent fluorescent signals produced from MB during the NESBA reaction containing (a) NASBA enzyme cocktail and (b) NE at varying concentrations in the presence (Red) and absence of

target gRNA (Black). The final concentrations of NASBA reaction buffer, NEBuffer™ 2.1, and a NESBA primer set are 1×, 0.5×, and 0.7 μM each, respectively.

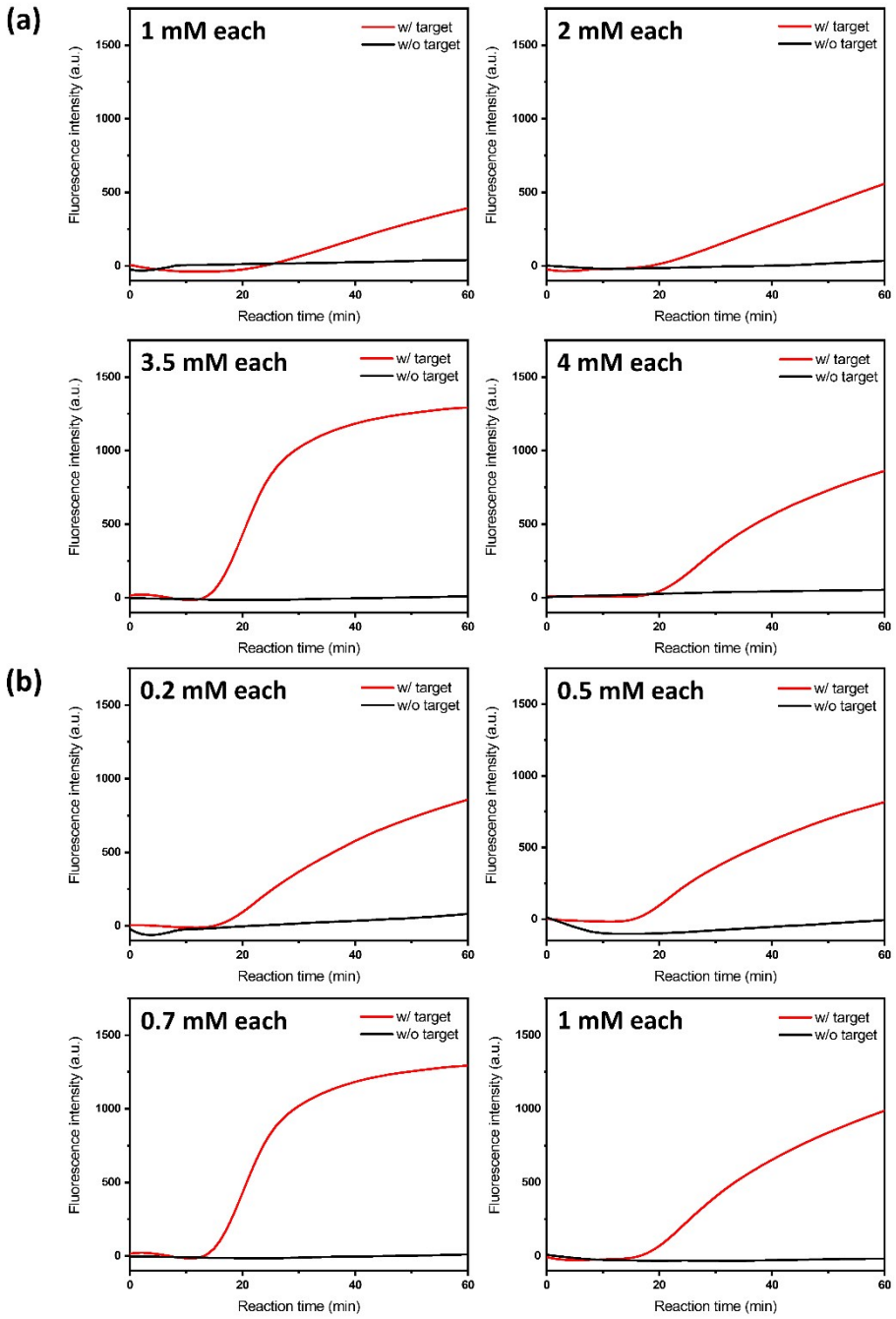

Fig. S3. Optimization of the rNTP and dNTP concentrations. Time-dependent fluorescent signals produced from MB during the NESBA reaction containing (a) rNTP and (b) dNTP at varying concentrations in the presence (Red) and absence of target gRNA (Black). The final

concentrations of NASBA reaction buffer, NEBuffer™ 2.1, NASBA enzyme cocktail, NE, and a NESBA primer set are 1×, 0.5×, 1×, 0.25 U/μL, and 0.7 μM each, respectively.

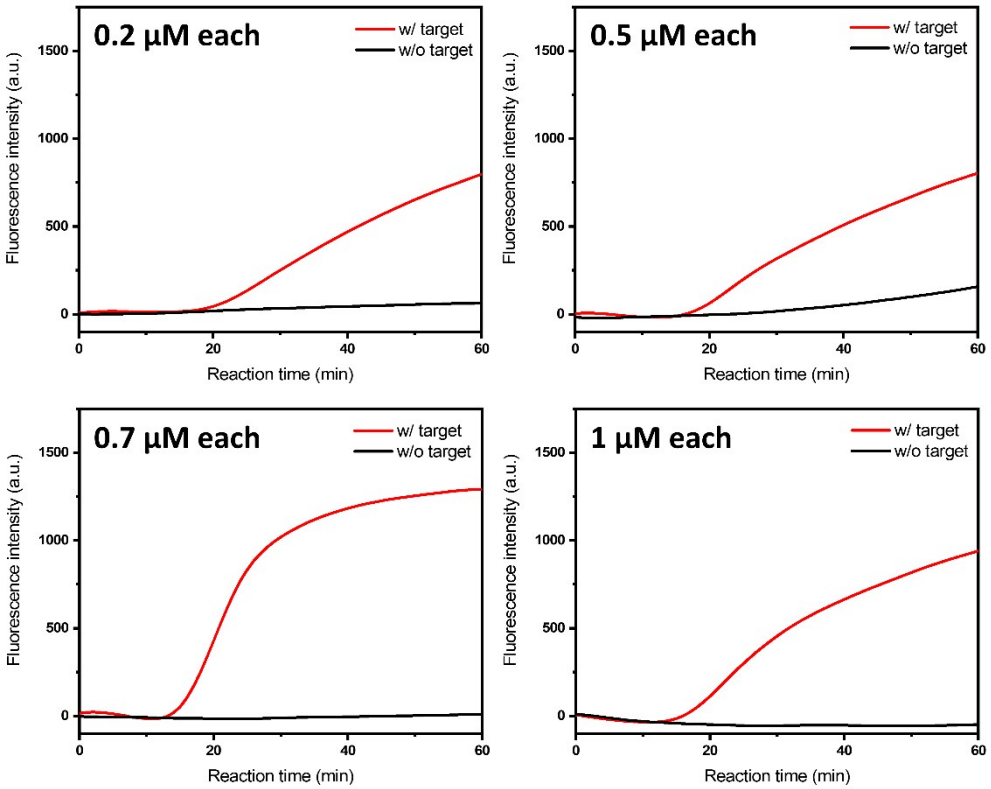

Fig. S4. Optimization of NESBA primer set concentration. Time-dependent fluorescent signals produced from MB during the NESBA reaction containing a NESBA primer set at varying concentrations in the presence (Red) and absence of target gRNA (Black). The final concentrations of NASBA reaction buffer, NEBuffer™ 2.1, NASBA enzyme cocktail, and NE are 1×, 0.5×, 1×, and 0.25 U/µL, respectively.

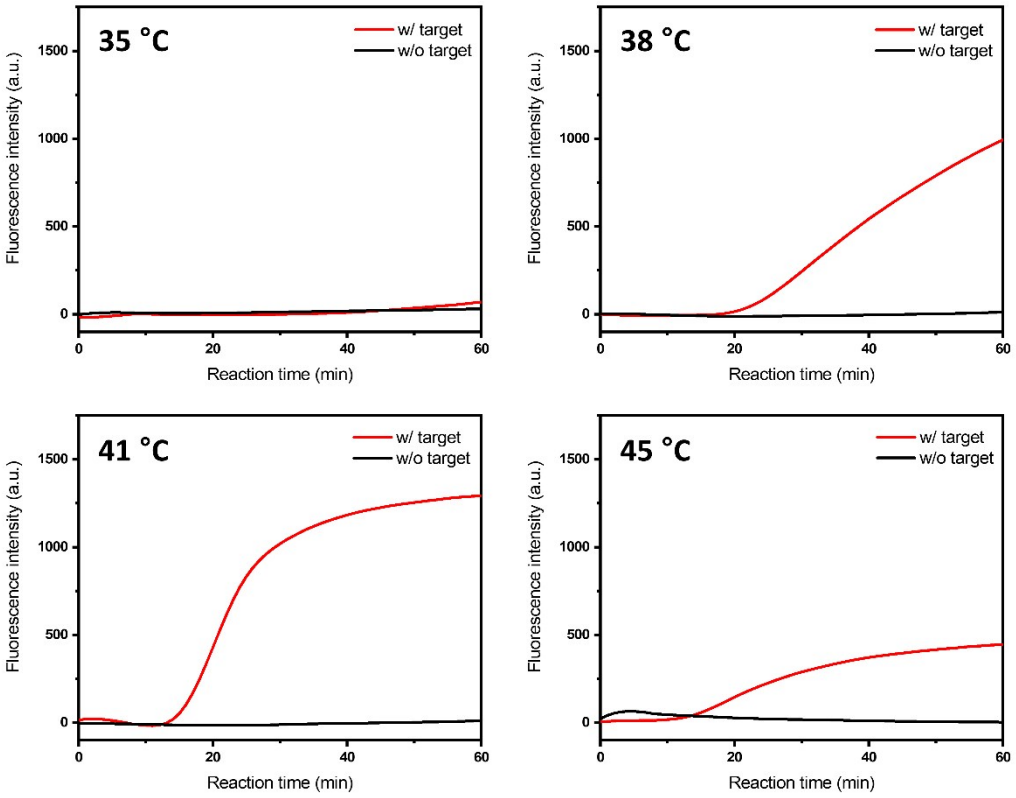

Fig. S5. Optimization of the reaction temperature. Time-dependent fluorescent signals produced from MB during the NESBA reaction in the presence (Red) and absence of target gRNA (Black) at various reaction temperatures. The final concentrations of NASBA reaction buffer, NEBuffer™ 2.1, NASBA enzyme cocktail, NE, and a NESBA primer set are 1×, 0.5×, 1×, 0.25 U/μL, and 0.7 μM each, respectively.

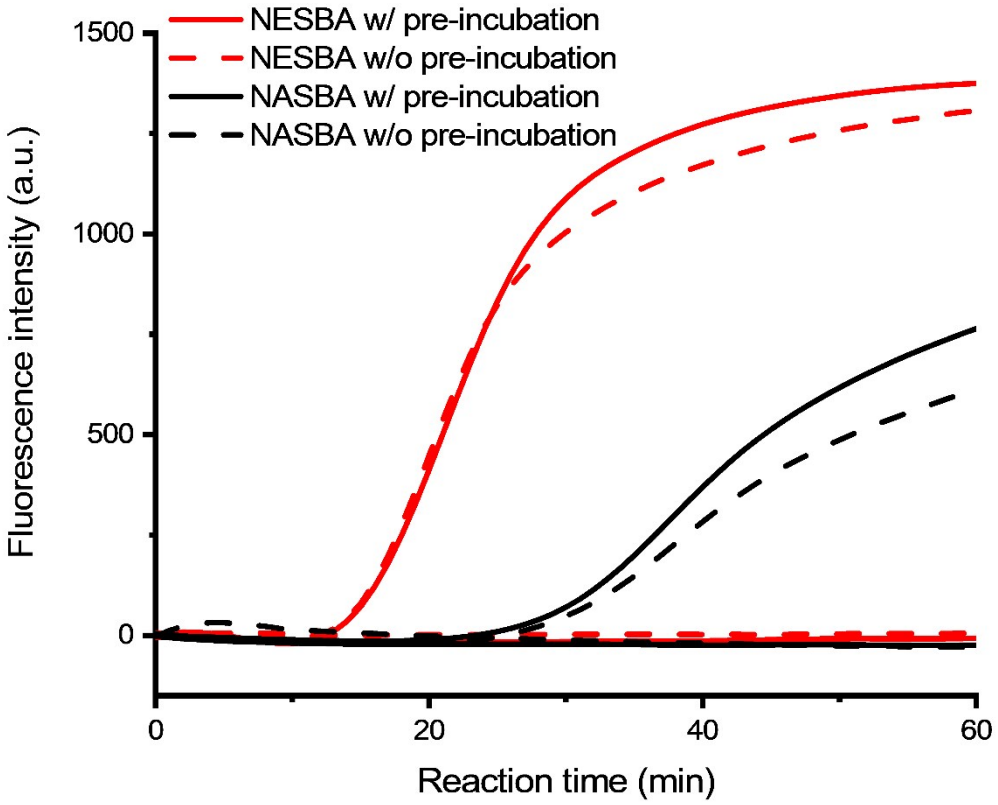

Fig. S6. The amplification efficiency of the NESBA and NASBA reactions with and without the initial denaturation step. Time-dependent fluorescent signals were produced from MB during the NESBA (red) and NASBA (black) reactions with (solid line) and without the initial denaturation step (dashed line). The final concentrations of NASBA reaction buffer, NEBuffer™ 2.1, NASBA enzyme cocktail, NE, and a NESBA/NASBA primer set are 1×, 0.5×, 1×, 0.25 U/μL, and 0.7 μM each, respectively.

Reference

C. Qian, R. Wang, H. Wu, F. Ji and J. Wu, *Anal. Chim. Acta*, 2019, **1050**, 1.