Supplementary Information

Non-Saturated Nucleic Acid Probes with a Broad Dynamic Range

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Content

Supporting Notes

1.1 Kinetic simulation of the NSNAP-III system

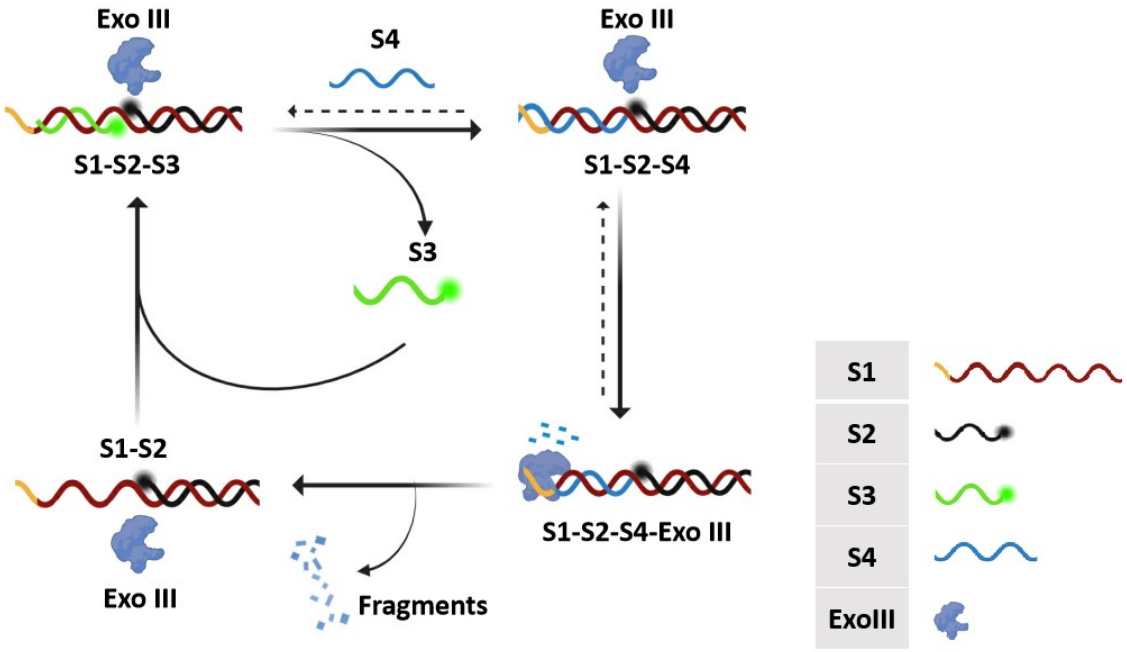

Figure 2D. Schematic of NSNAP-III operation

The reaction equations are as follows：

(1). $S4 + S1 - S2 - S3 \overset{kI}{\rightarrow} S1 - S2 - S4 + S3$

(2). $S1 - S2 - S4 + ExoIII \underset{kIII}{\overset{kII}{\rightleftharpoons}} S1 - S2 - S4 - ExoIII$

(3). $S1 - S2 - S4 - ExoIII \overset{kIV}{\rightarrow} S3 + ExoIII + Fragments$

(4). $S1 - S2 + S3 \overset{kV}{\rightarrow} S1 - S2 - S3$

The differential equations are as follows：

dydx[0] = k_V × y[3] × y[4] - k_I × y[0] × y[6];

dydx[1] = k_I × y[0] × y[6] + k_{III} × y[2] - k_{II} × y[1] × y[5];

dydx[2] = k_{II} × y[1] × y[5] - k_{III} × y[2] - k_{IV} × y[2] ;

dydx[3] = k_{IV} × y[2] - k_V × y[3] × y[4] ;

dydx[4] = k_I × y[0] × y[6] - k_V × y[3] × y[4] ;

dydx[5] = k_{III} × y[2] + k_{IV} × y[2] - k_{II} × y[1] × y[5] ;

dydx[6] = 0 - k_I × y[0] × y[6] ;

dydx[7] = k_{IV} × y[2] .

The notations in Eq.:

Species	Kinetic parameter
0 = S1-S2-S3	k_I **=3.1×10^5 M^{-1} s^{-1}**
1 = S1-S2-S4	k_{II} **=1.4×10^7 M^{-1} s^{-1}**
2 = S1-S2-S4-ExoIII	k_{III} **=0.0027 s^{-1}**
3 = S1-S2	k_{IV} **=0.41 s^{-1}**
4 = S3	k_V **=5.735×10^5 M^{-1} s^{-1}**
5 = ExoIII	
6 = S4	
7 = Fragments	

The differential equations are set up observing the following assumptions:

(1) Enzyme kinetics should be observed to follow the Michaelis-Menten equation;

(2) That the TMSD reaction and the enzyme kinetic reaction are carried out independently and do not affect each other;

(3) The strand rehybridization process is an irreversible reaction;

(4) Enzymatic digestion of the target to produce dNMP does not affect the whole reaction process.

Kinetic simulation of the NSNAP-III system:

In the NSNAP-III system, the change of S3 concentration was recorded as the fluorescence kinetics of the reaction. During the reaction, the generation of S3 represents the fluorescence rise, and the refolding of S3 and S1-S2 to form S1-S2-S3 represents the fluorescence fall.

The designed Toehold length of S1-S2-S3 is 6 nt without reverse toehold, which is considered as an irreversible reaction. Based on the model developed, the rate constants of TMSD used for simulation were predicted. When the TMSD was irreversible, k_I was predicted to be 3.1 × 10^5 M^{-1} s^{-1} when the Toehold length was 6 nt. The Michaelis–Menten kinetics constants K_m and *k*cat for the simulation were

experimentally determined: K_m was 29.48 nM, and *k*cat was 0.41 s^{-1}. The Exo III concentration was converted to an apparent molar concentration, e.g., 250 U/ml was 53.57 nM. We assumed at least 95% binding of the enzyme to the substrate (enzyme saturation regime) when the substrate was in excess. Thus, according to reaction (2) in Supporting note 1, k_{II}/k_{III}=95%/5%×[S1-S2-S4], where we used 200 nM for S1-S2-S4. Based on the Michaelis–Menten kinetics model, $K_m = (k_{III} + k_{IV})/k_{II}$, k_{II} and k_{III} were solved as 1.4×10^7 M^{-1} s^{-1} and 0.0027 s^{-1}, respectively. k_V for the simulation was determined to be 5.735×10^5 $M^{-1}s^{-1}$ by fitting the experimental data.

1.2 Kinetic simulation of the NSNAP-λ system

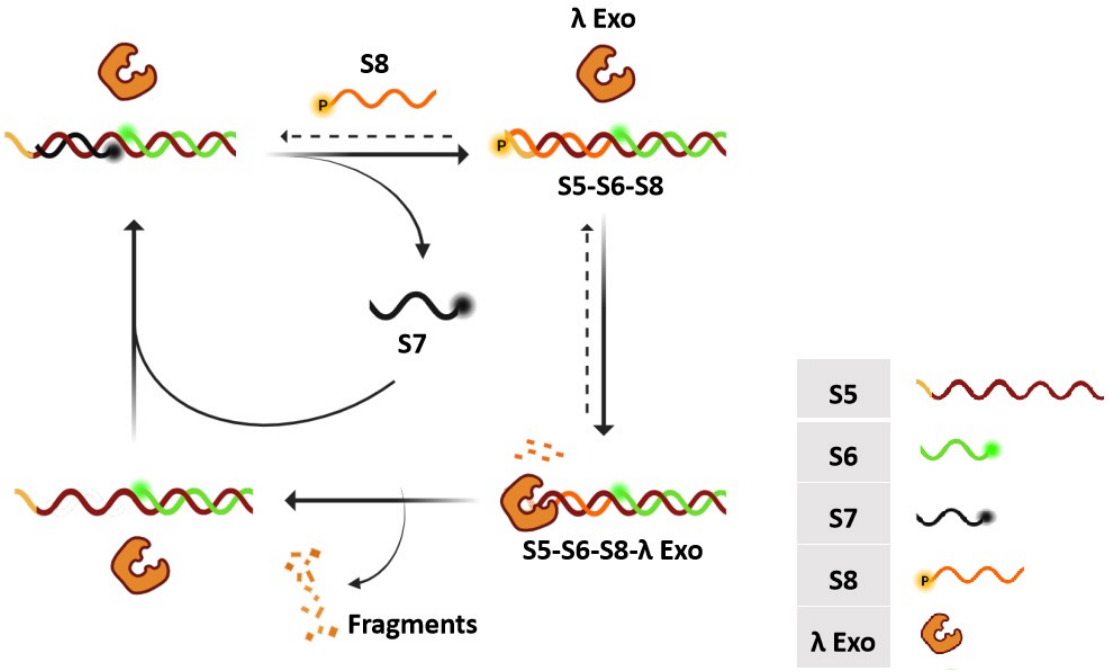

Figure 3A. Schematic of NSNAP-λ operation

The reaction equations are as follows:

$$S8 + S5-S6-S7 \underset{kVII}{\overset{kVI}{\rightleftharpoons}} S5-S6-S8 + S7 \quad (1).$$

$$S5-S6-S8 + \lambda Exo \underset{kIX}{\overset{kVIII}{\rightleftharpoons}} S5-S6-S8-\lambda Exo \quad (2).$$

$$S5-S6-S8-\lambda Exo \xrightarrow{kX} S5-S6 + \lambda Exo + Fragments \quad (3).$$

(4).$S7 + S5 - S6 \overset{kXI}{\rightarrow} S5 - S6 - S7$

The differential equations are as follows:

dydx[0] = k_X × y[6] - k_{XI} × y[0] × y[1];

dydx[1] = k_{VI} × y[4] × y[3] - k_{VII} × y[5] × y[1] - k_{XI} × y[0] × y[1];

dydx[2] = k_{IX} × y[6] + k_X × y[6] - k_{VIII} × y[5] × y[2];

dydx[3] = k_{VII} × y[5] × y[1] - k_{VI} × y[4] × y[3];

dydx[4] = k_{XI} × y[0] × y[1] + k_{VII} × y[5] × y[1] - k_{VI} × y[4] × y[3];

dydx[5] = k_{VI} × y[4] × y[3] + k_{IX} × y[6] - k_{VII} × y[5] × y[1] - k_{VIII} × y[5] × y[2];

dydx[6] = k_{VIII} × y[5] × y[2] - k_{IX} × y[6] - k_X × y[6].

The notations in Eq.：

Species	Kinetic parameter
0 = S5-S6	**k_{VI} = 0.0138 M^{-1} s^{-1}**
1 = S7	**k_{VII} = 5.27E-25 M^{-1} s^{-1}**
2 = λExo	**k_{VIII} = 0.001159 M^{-1} s^{-1}**
3 = S8	**k_{IX} = 0.003048 s^{-1}**
4 = S5-S6-S7	**k_X = 0.304 s^{-1}**
5 = S5-S6-S8	**k_{XI} = 0.004389 M^{-1} s^{-1}**
6 = S5-S6-S8-λExo	

Kinetic simulation of the NSNAP-λ system:

In the NSNAP-λ system, the change in the concentration of S5-S6 was recorded as the fluorescence kinetics of the reaction. During the reaction, the generation of S5-S6 resulted in an increase in fluorescence, while the refolding of S5-S6 and S7 to form S5-S6-S7 led to a decrease in fluorescence.

1.3 Calculation of Area Under the Curve (AUC) for Fluorescence Intensity Data

The fluorescence intensity data are collected over time, and the AUC is computed using numerical integration methods. The formula used for calculating AUC can be expressed as:

$$\mathrm{AUC} = \sum_{i=1}^{n-1} \frac{(x_{i+1} - x_i) \cdot (y_i + y_{i+1})}{2}$$

Where y_{i+1} and y_i are the fluorescence intensities of adjacent data points, and x_{i+1} and x_i are the corresponding time point.

Supporting Figures

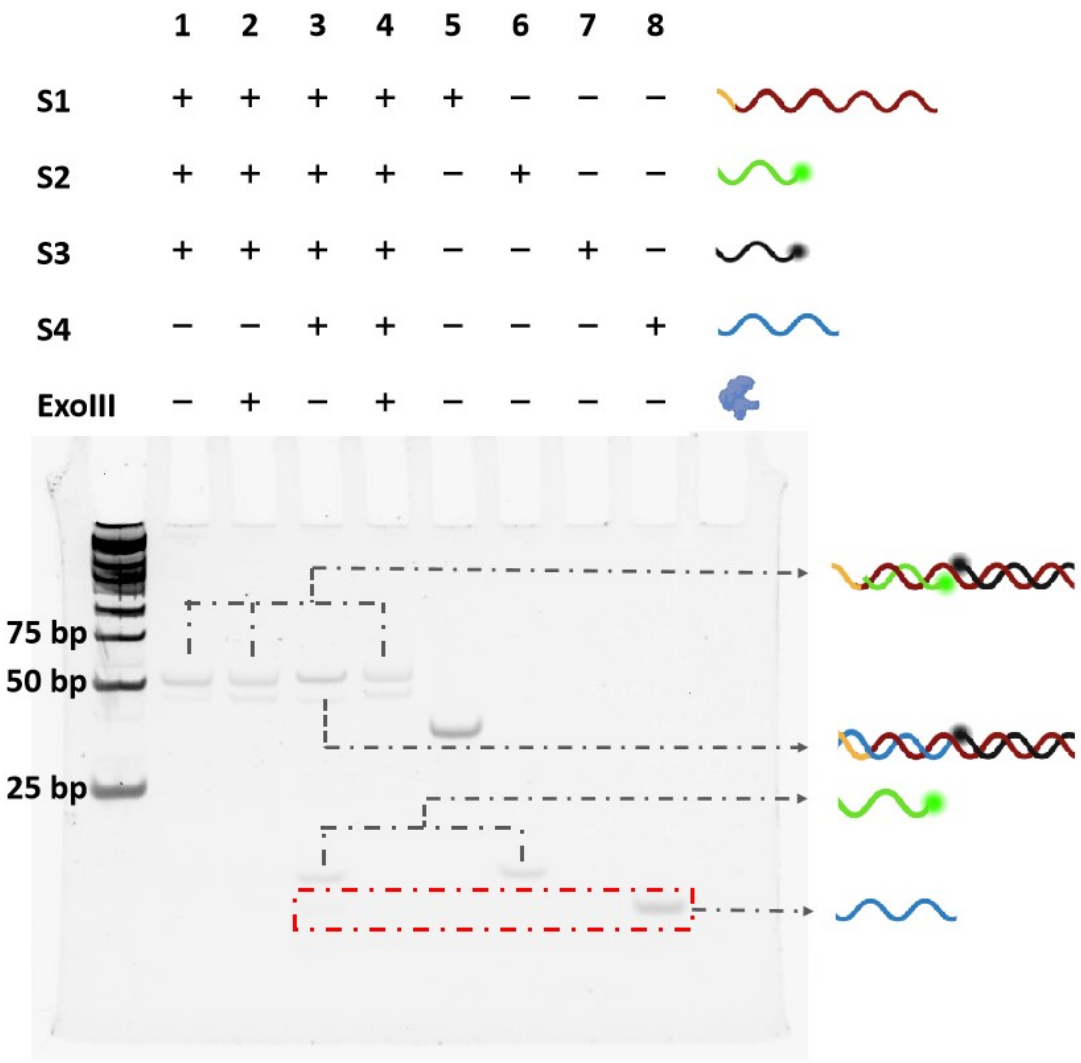

Figure S1. Gel electrophoretic characterization of the reaction between NSNAP-III and the target strand. Lane 1: nucleic acid probe. Lane 2: NSNAP-III. Lane 3: probe-target complex, which was formed by the strand displacement reaction between the target strand and the nucleic acid probe, resulting in the release of the fluorescent strand. Lane 4: NSNAP-III after being reset following complete target depletion. The target strand band is highlighted with a red box. All reactions were performed at 37 ℃ with a nucleic acid probe concentration of 200 nM, Exo III at 250 U/mL, and NEBuffer 1 as the reaction buffer. The gel electrophoresis protocol is described in detail in Experimental Section 2.4.

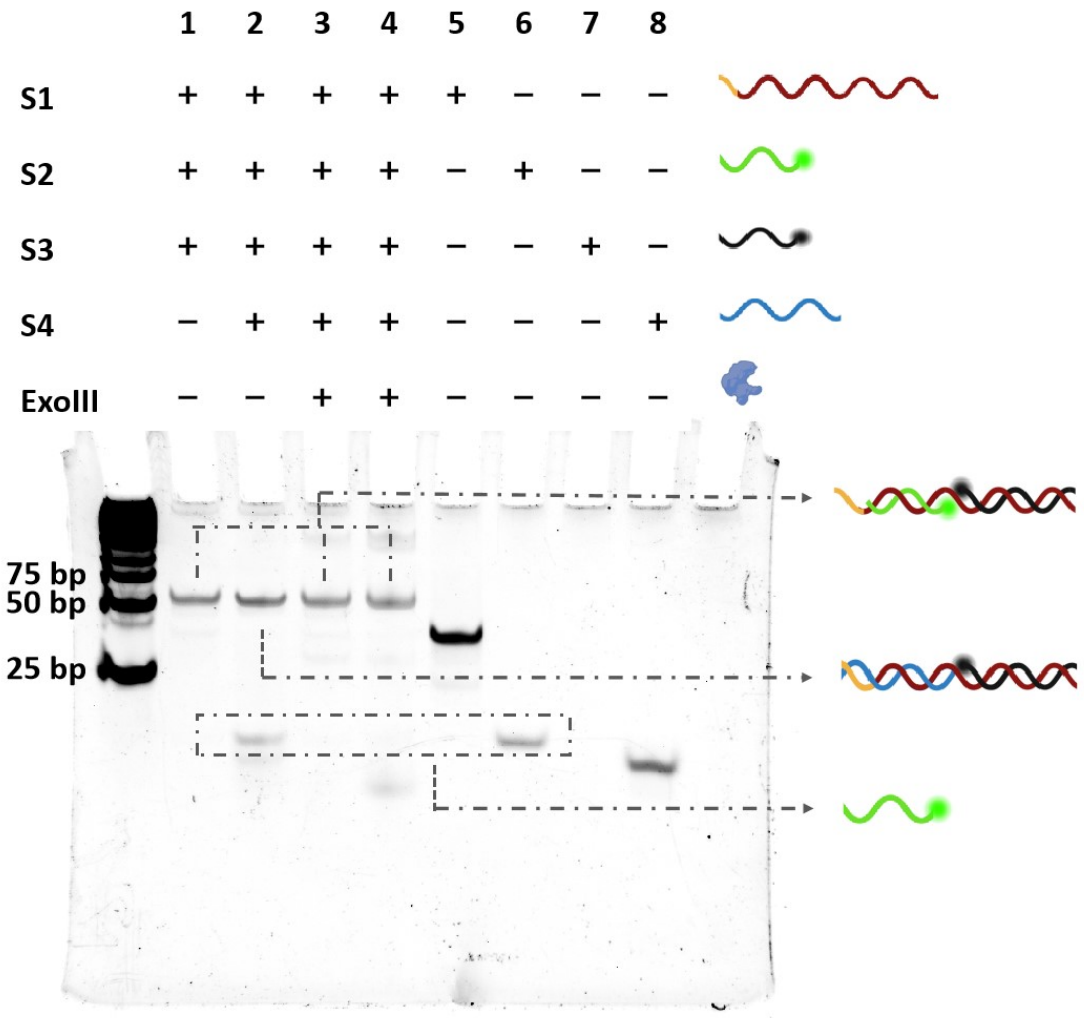

Figure S2. Gel electrophoretic characterization of verifying probe stability during repeated cycles. Lane 1: nucleic acid probe. Lane 2: probe-target complex, which was formed by the strand displacement reaction between the target strand and the nucleic acid probe, resulting in the release of the fluorescent strand. Lane 3: NSNAP-III reset after one cycle. Lane 4: NSNAP-III reset after seven cycles. All reactions were performed at 37 ℃ with a nucleic acid probe concentration of 200 nM, Exo III at 250 U/mL, and NEBuffer 1 as the reaction buffer. The gel electrophoresis protocol is described in detail in Experimental Section 2.4.

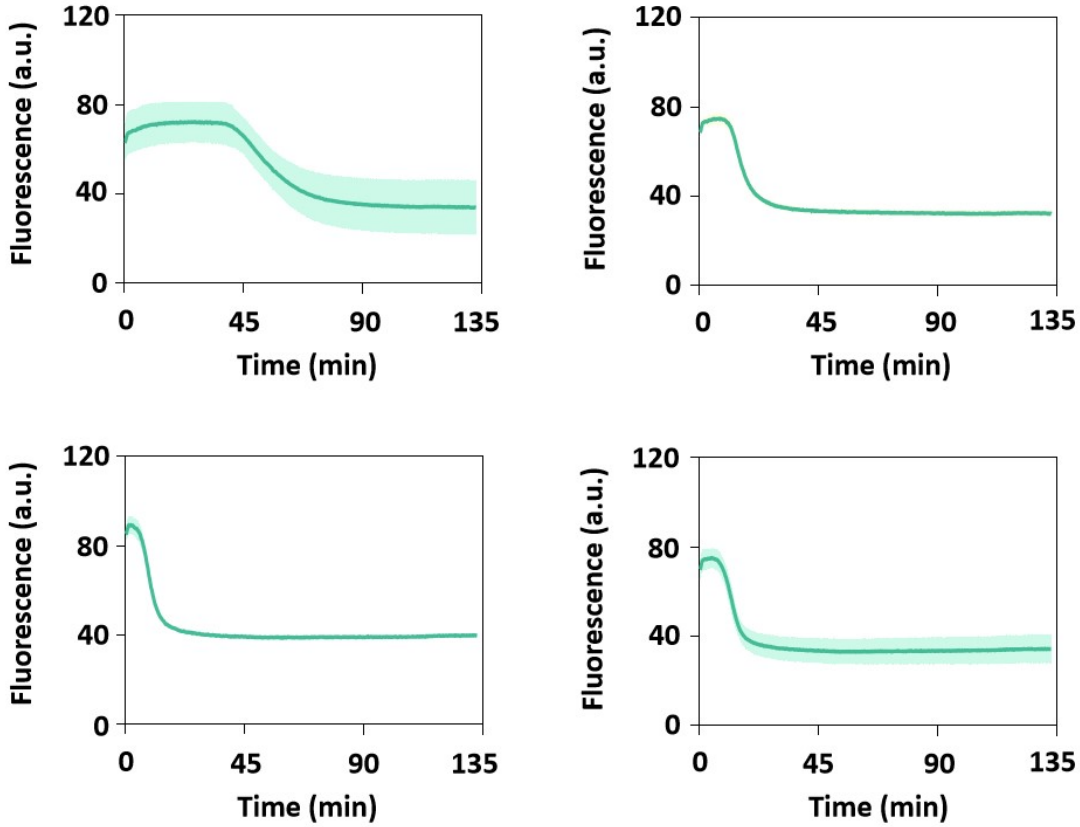

Figure S3. Fluorescence kinetic curves for NSNAP-III detection of 20 μM target at varying Exo III concentrations (50 U/mL, 150 U/mL, 250 U/mL, 500 U/mL). The nucleic acid probe concentration was 200 nM in all experiments, and NEBuffer 1 was used as the reaction buffer. All experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

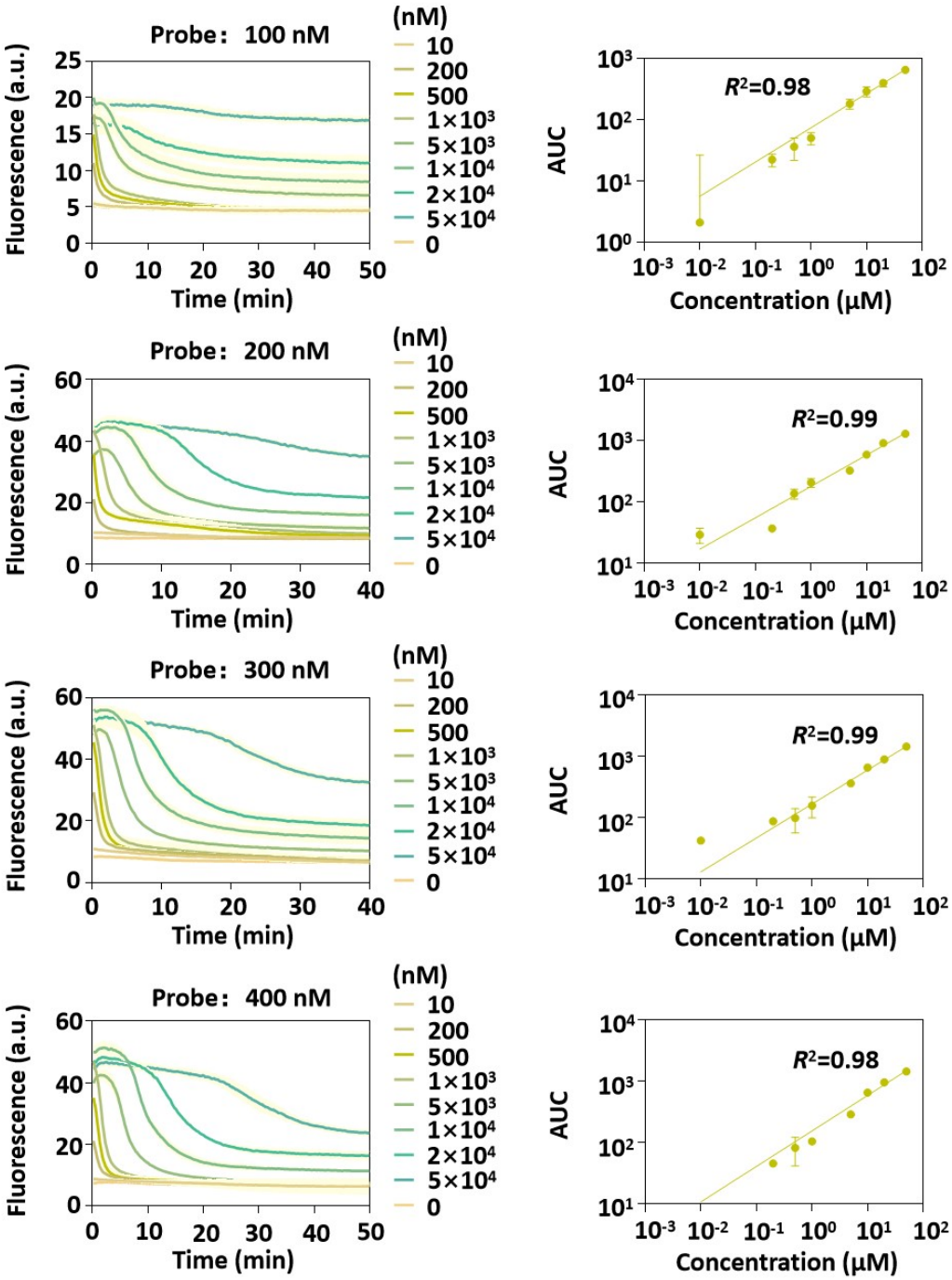

Figure S4. Fluorescence kinetic curves of NSNAP-III constructed with different concentrations of nucleic acid probes (100 - 400 nM) reacting with targets spanning concentrations from 10 nM to 50 μM. A linear relationship was observed between the area under the curve and target concentration, with R^2 values of 0.98, 0.99, 0.99, and 0.98, respectively. In all experiments, the Exo III concentration was 250 U/mL, and NEBuffer 1 was used as the reaction buffer. Experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

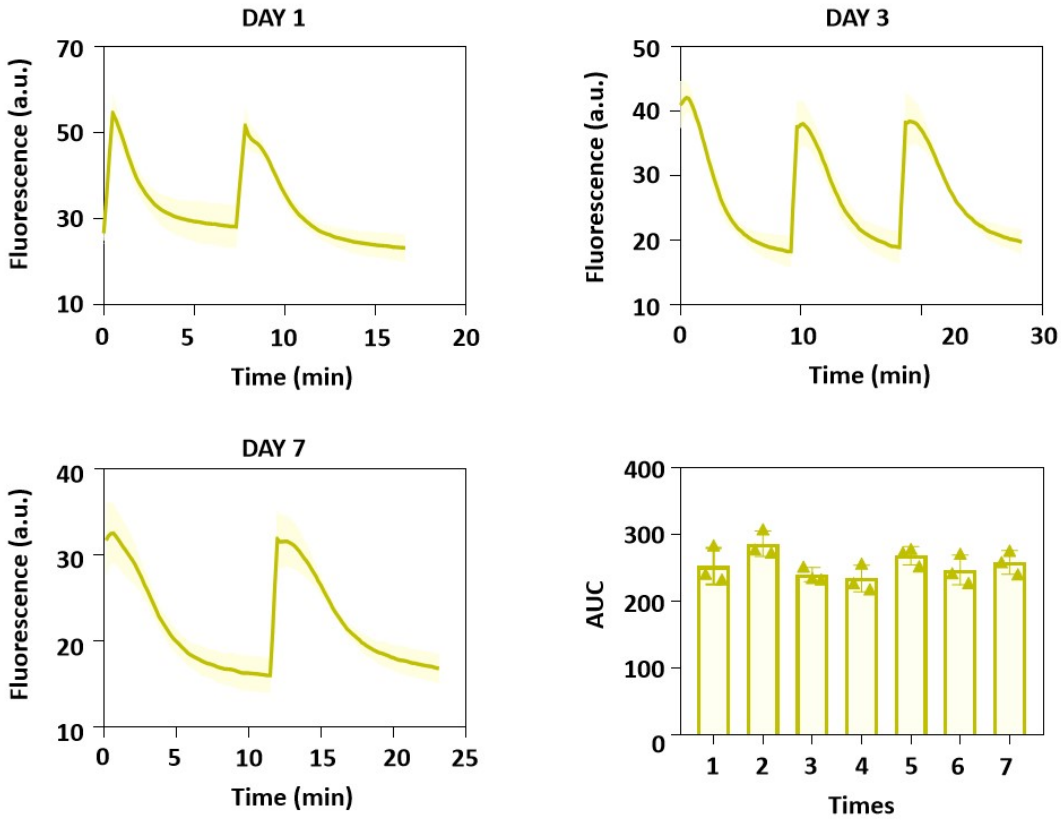

Figure S5. Fluorescence kinetic curves generated by NSNAP-III reused seven times over the course of one week, along with the corresponding area under the curve for each reuse (nucleic acid probe concentration: 200 nM, target concentration: 1 μM). NSNAP-III was stored at 4 ℃ between uses. In all experiments, the Exo III concentration was 250 U/mL, and NEBuffer 1 was used as the reaction buffer. Experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

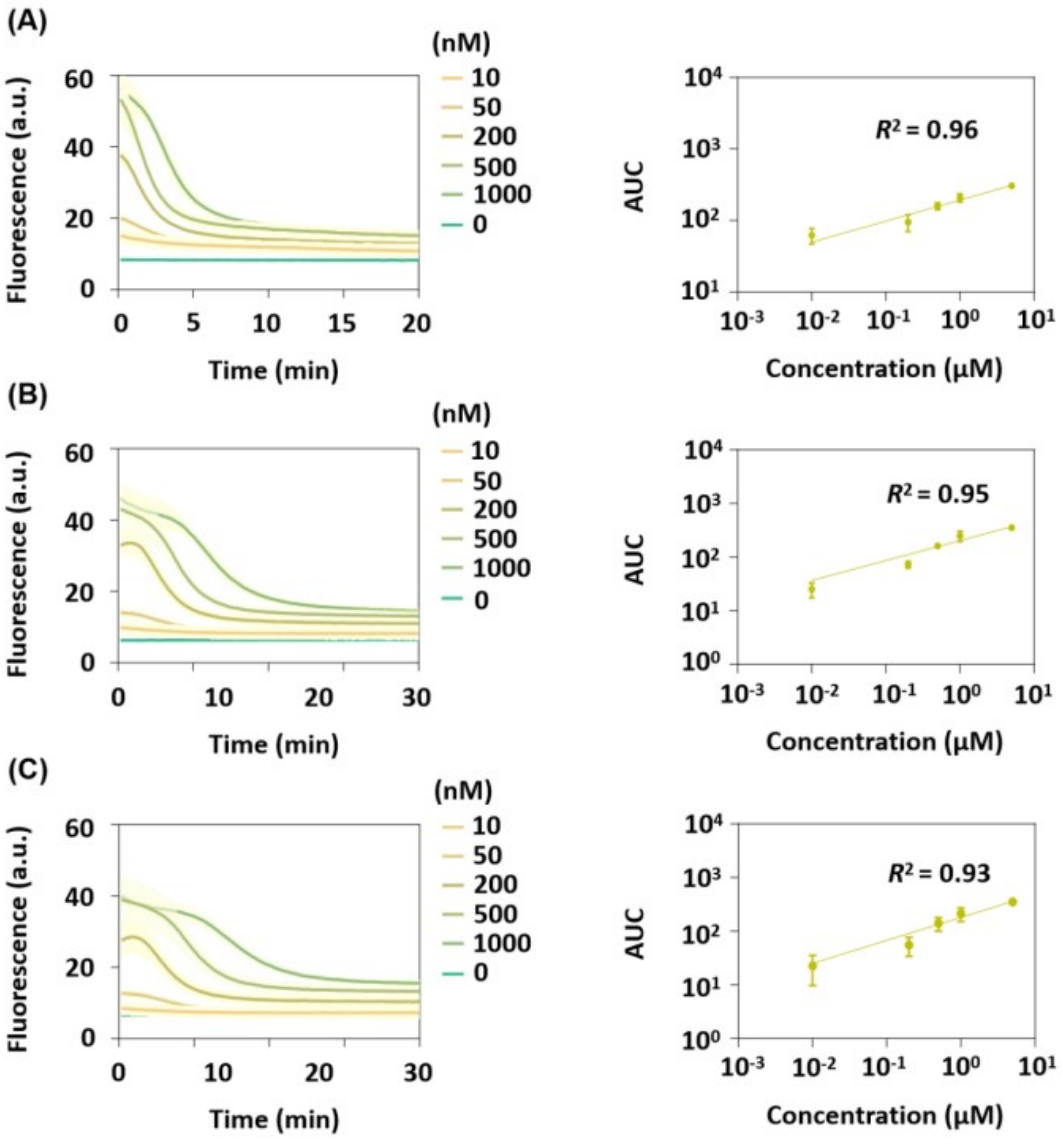

Figure S6. Fluorescence kinetics of the NSNAP-III detection system after long-term storage. (A) First use: real-time fluorescence curves (left) and linear relationship between the area under the fluorescence kinetic curve and target concentration (right; R^2 = 0.96). (B) After storage at 4 ℃ for 3 days: freal-time fluorescence curves (left) and linear relationship between the area under the fluorescence kinetic curve and target concentration (right; R^2 = 0.95). (C) After storage at 4 ℃ for 7 days: real-time fluorescence curves (left) and linear relationship between the area under the fluorescence kinetic curve and target concentration (right; R^2 = 0.93) .NSNAP-III was prepared with 200 nM nucleic acid probe and 250 U/mL Exonuclease III in NEBuffer 1 at 37 ℃, and target concentrations of 10-1000 nM were detected. Data are the means ± s.d. (n = 3 independent experiments)

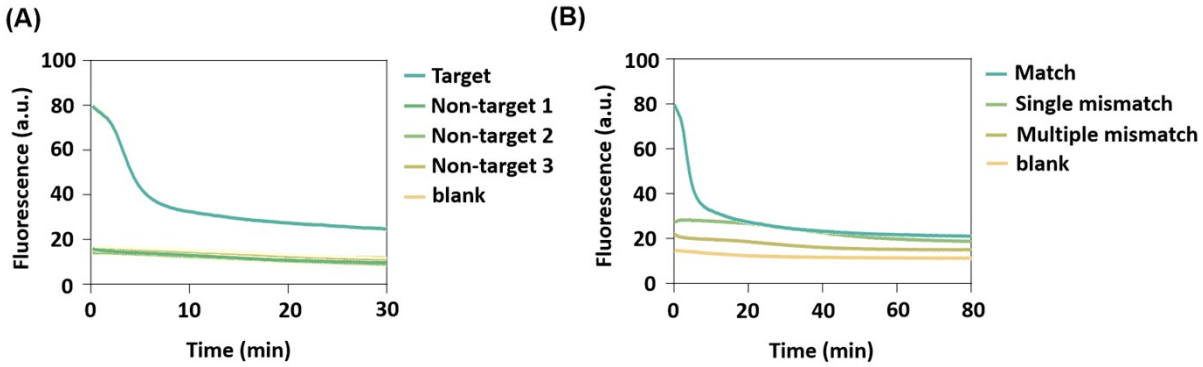

Figure S7. Verification of the specificity of NSNAP-III and its sensitivity to single base mutations. (A) Fluorescent signals generated by NSNAP-III in detecting target and non-target sequences. (B) Fluorescent signals generated by NSNAP-III in detecting matched, single-mismatched, and multiple-mismatched sequences. The nucleic acid probe concentration was 200 nM, the target concentration was 2 μM, and the Exo III concentration used in all experiments was 250 U/mL, with NEBuffer 1 at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

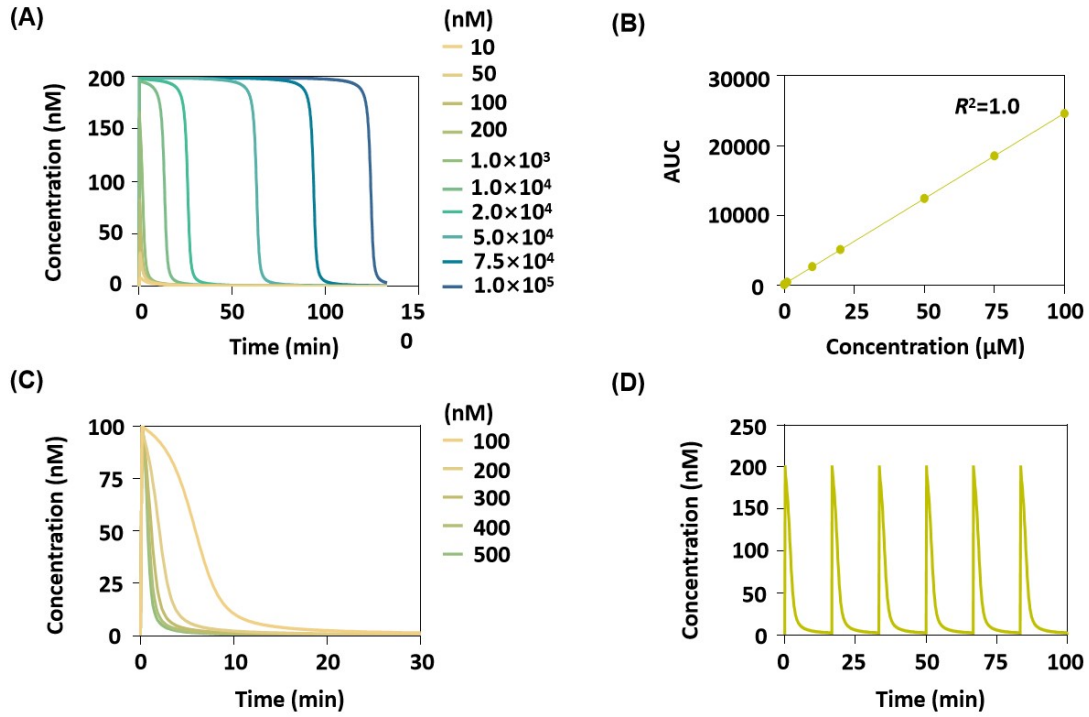

Figure S8. Kinetic simulation of NSNAP-III reaction with the target. (A) Simulated fluorescence curves for NSNAP-III (nucleic acid probe concentration: 200 nM) detecting target concentrations ranging from 10 nM to 100 μM. The modeling process is detailed in the Supporting Note, with sequences provided in Supporting Table S1. (B) Simulated area under the kinetic curve for NSNAP-III detection of targets as a function of target concentration, demonstrating a linear relationship with R^2 = 1.0. (C) Normalized fluorescence signals from the kinetic simulation of NSNAP-III (nucleic acid probe concentrations: 100 - 500 nM) reacting with 1 μM target. (D) Simulated kinetic signals for NSNAP-III reused seven times (nucleic acid probe concentration: 200 nM, target concentration: 1 μM). In all simulations, the Exo III concentration was set to 250 U/mL

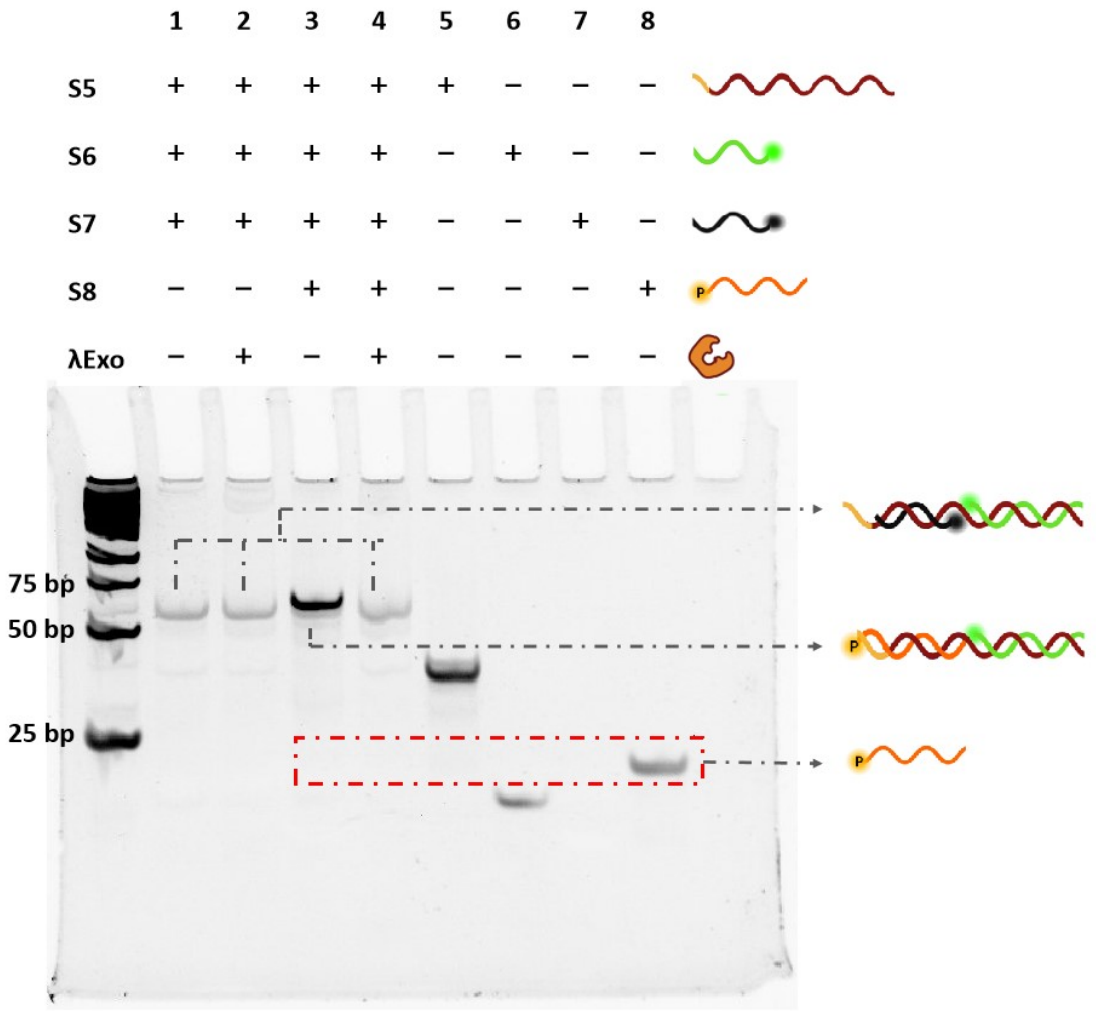

Figure S9. Gel electrophoresis characterization of the reaction between NSNAP-λ and the target strand. Lane 1: nucleic acid probe. Lane 2: NSNAP-λ. Lane 3: probe-target complex formed through the strand displacement reaction between the target strand and the probe. Lane 4: NSNAP-λ after being reset following complete target depletion. The target strand band is highlighted with a red box. All reactions were conducted at 37 °C using a nucleic acid probe concentration of 500 nM, λ Exo at 500 U/mL, and Lambda Exonuclease Reaction Buffer. Details of the gel electrophoresis protocol are provided in Experimental Section 2.4.

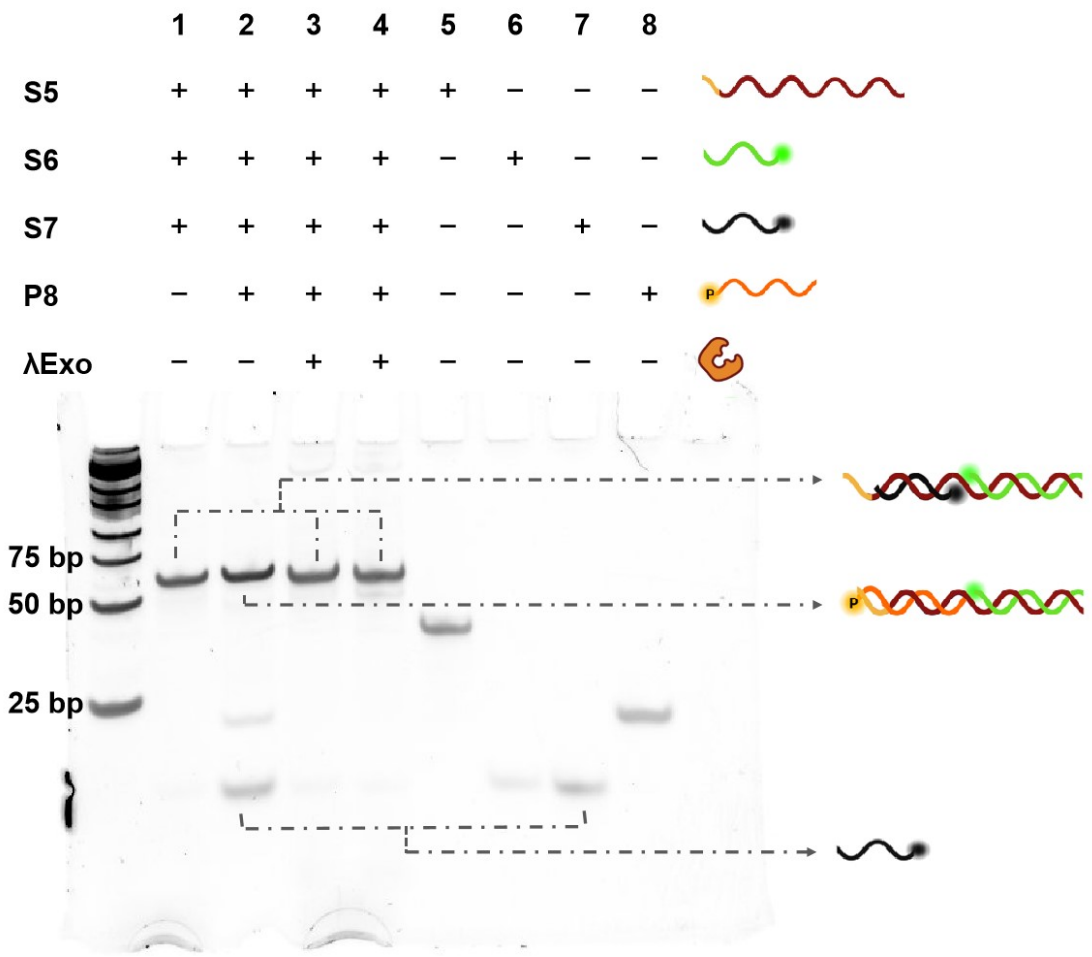

Figure S10. Gel electrophoretic characterization of confirming probe stability during repeated cycles. Lane 1: nucleic acid probe. Lane 2: probe-target complex, which was formed by the strand displacement reaction between the target strand and the nucleic acid probe, resulting in the release of the fluorescent strand. Lane 3: NSNAP-λ reset after one cycle. Lane 4: NSNAP-λ reset after seven cycles. All reactions were conducted at 37 ℃ using a nucleic acid probe concentration of 500 nM, λ Exo at 500 U/mL, and Lambda Exonuclease Reaction Buffer. Details of the gel electrophoresis protocol are provided in Experimental Section 2.4.

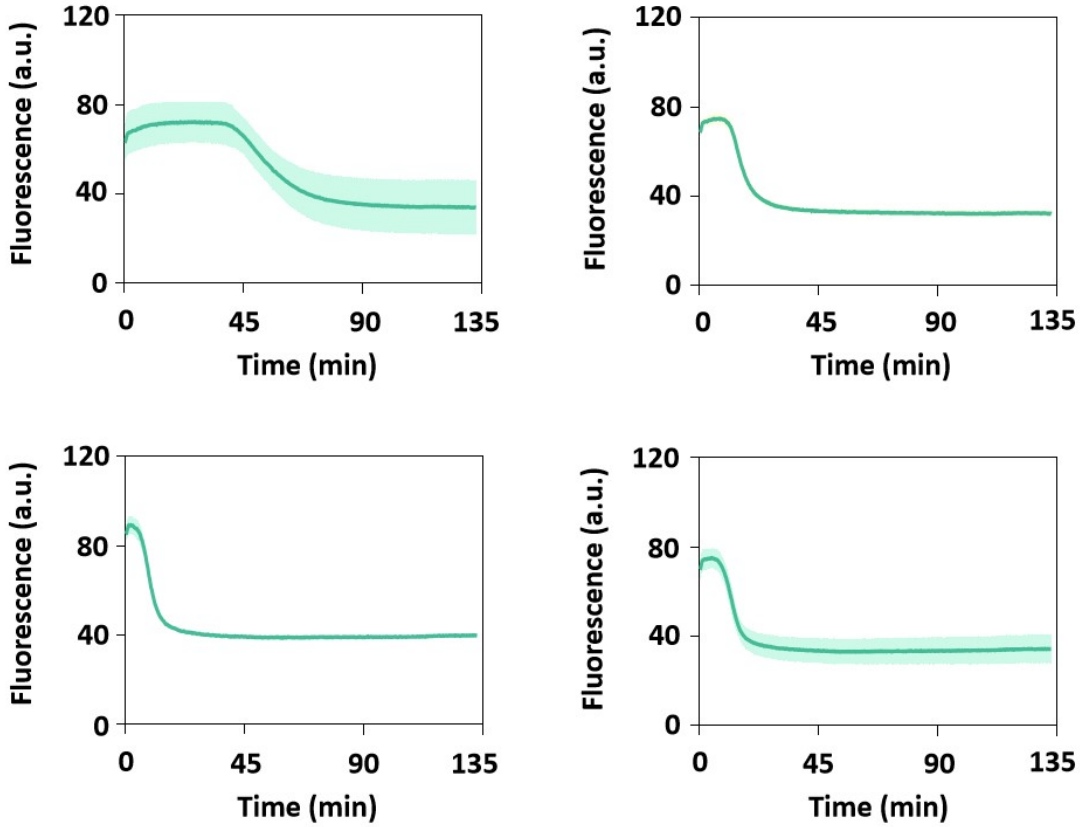

Figure S11. Fluorescence kinetic curves for NSNAP-λ detection of 10 μM targets at varying λ Exo concentrations (50 U/mL, 100 U/mL, 250 U/mL, 500 U/mL). The nucleic acid probe concentration was 500 nM, and the reaction buffer used was Lambda Exonuclease Reaction Buffer. All experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

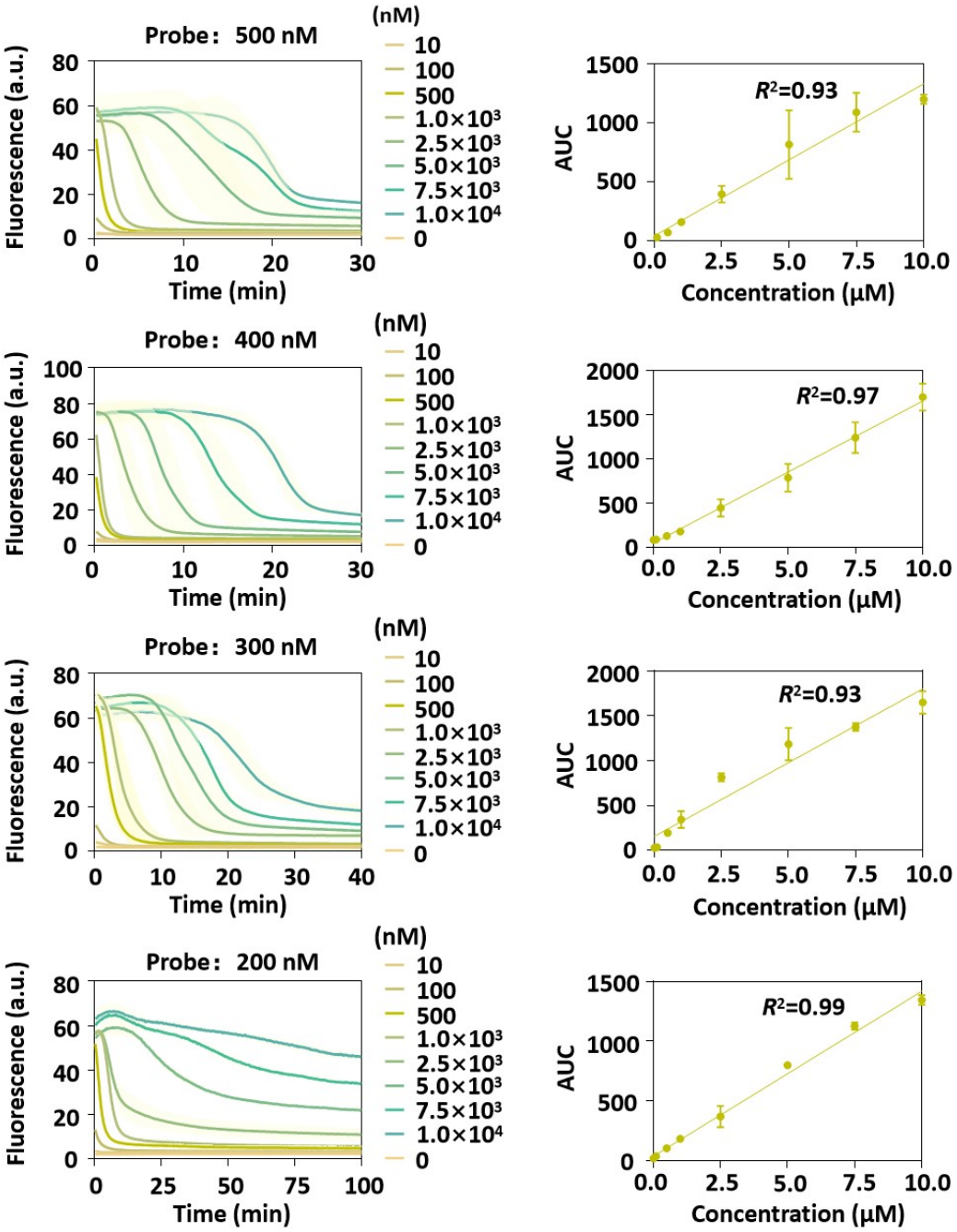

Figure S12. Fluorescence kinetic curves of NSNAP-λ constructed with different concentrations of nucleic acid probes (500 - 200 nM) reacting with target concentrations ranging from 10 nM to 10 μM. A linear relationship was observed between the area under the curve and target concentration, with R^2 values of 0.93, 0.97, 0.93, and 0.99, respectively. All experiments used λ Exo at a concentration of 500 U/mL, with Lambda Exonuclease Reaction Buffer as the reaction medium. Experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

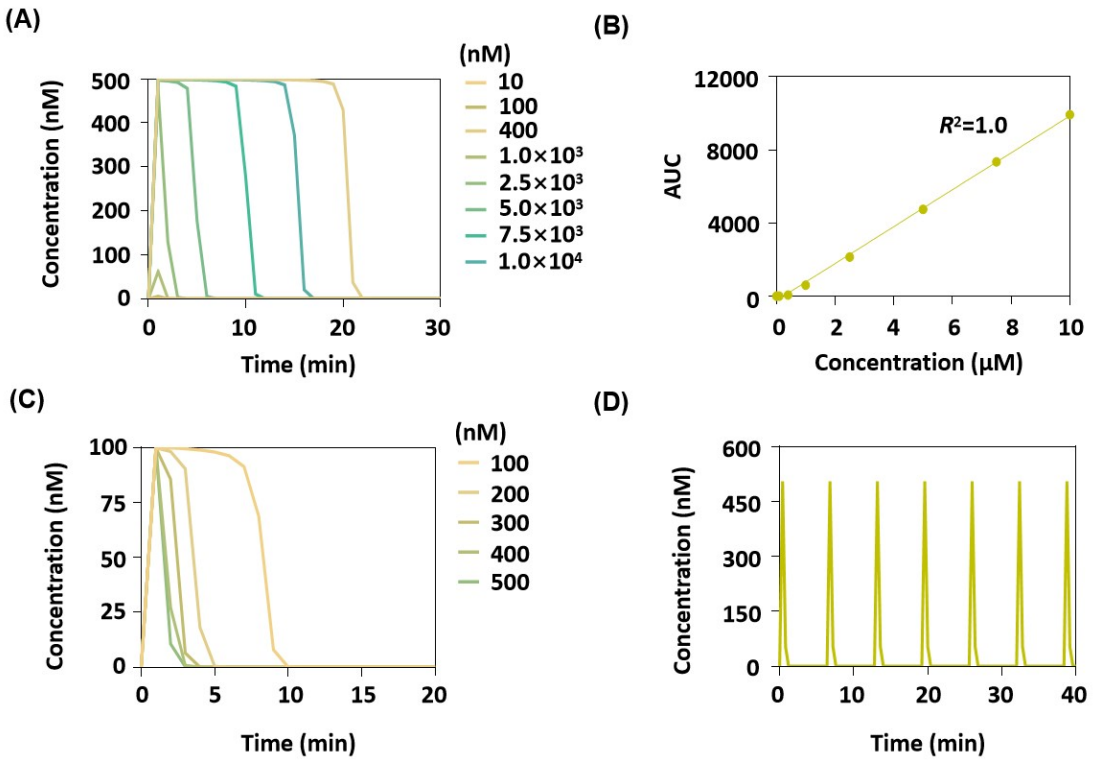

Figure S13. Kinetic simulation of NSNAP-λ reaction with the target. (A) Simulated kinetic curves for NSNAP-λ (nucleic acid probe concentration: 500 nM) detecting targets at various concentrations (10 nM - 10 μM). The modeling process is described in the Supporting Note, with sequences provided in Supporting Table S2. (B) Linear relationship between the area under the kinetic curve and target concentration, with R^2 = 1.0. (C) Normalized signals generated by simulating the reaction of NSNAP-λ at different nucleic acid probe concentrations (100 - 500 nM) with a 1 μM target. (D) Simulated kinetic signals for NSNAP-λ reused seven times (nucleic acid probe concentration: 500 nM, target concentration: 1 μM). In all simulations, the λ Exo concentration was set to 500 U/mL.

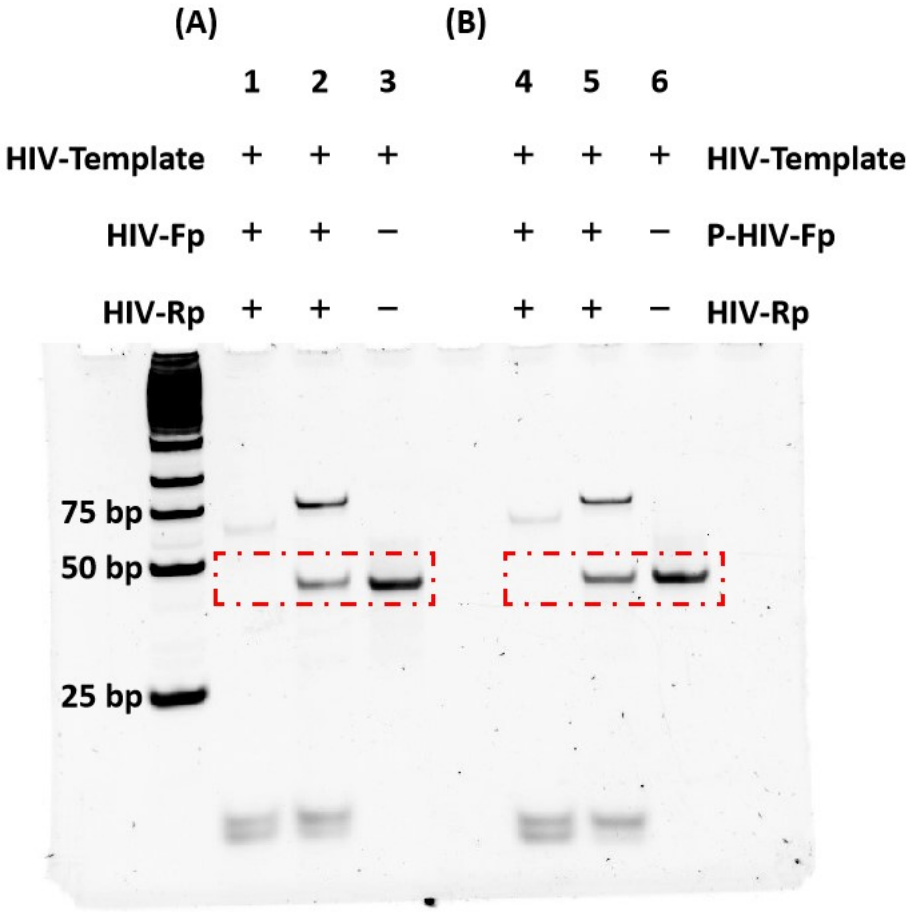

Figure S14. Gel electrophoresis characterization of LATE-PCR products of the HIV gene. (A) NSNAP-III detection system. Lane 1 shows the HIV gene template along with upstream and downstream primers, and Lane 2 shows the amplification product. (B) NSNAP-λ detection system. Lane 4 shows the HIV gene template and primers, and Lane 5 shows the amplification product. The single-stranded target was successfully amplified, with the target strand band highlighted in red. The template strand concentration was 1000 fM. Details of the gel electrophoresis characterization are provided in Experimental Section 2.4, and the LATE-PCR experiment is described in Experimental Section 2.5.

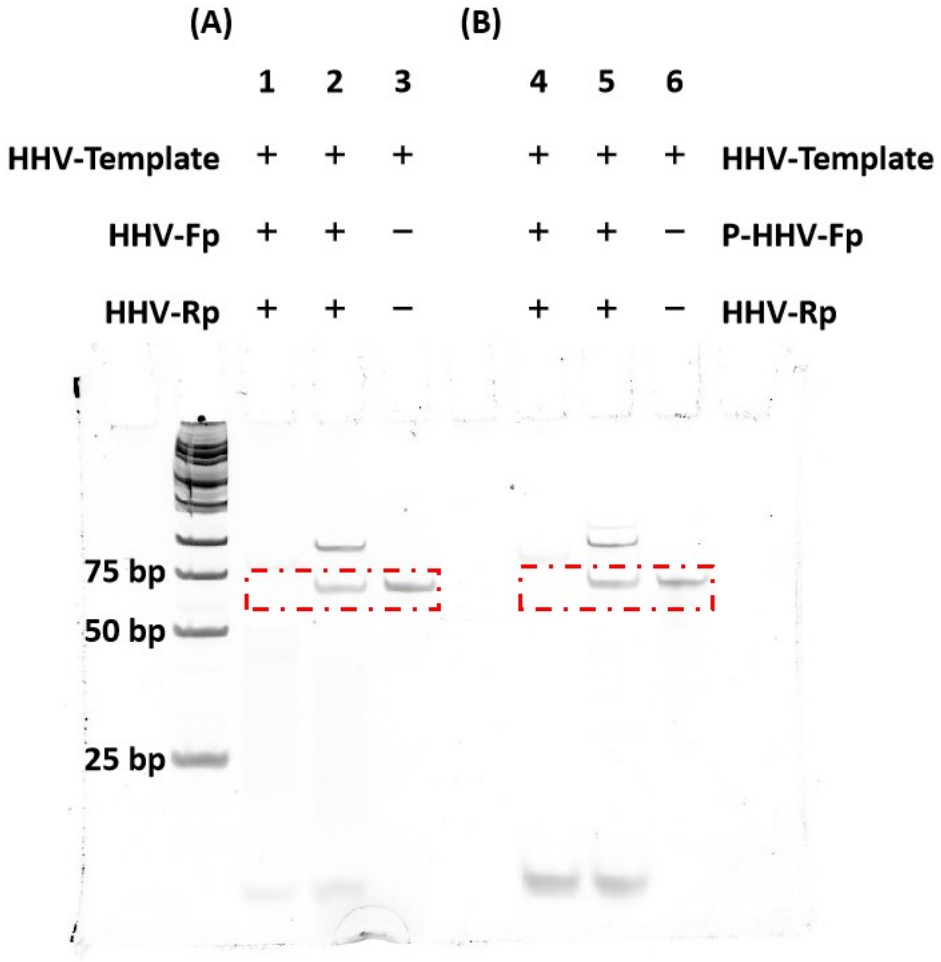

Figure S15. Gel electrophoresis characterization of LATE-PCR products of the HHV gene. (A) NSNAP-III detection system. Lane 1 shows the HHV gene template along with upstream and downstream primers, and Lane 2 shows the amplification product. (B) NSNAP-λ detection system. Lane 4 shows the HHV gene template and primers, and Lane 5 shows the amplification product. The single-stranded target was successfully amplified, with the target strand band highlighted in red. The template strand concentration was 1000 fM. Details of the gel electrophoresis characterization are provided in Experimental Section 2.4, and the LATE-PCR experiment is described in Experimental Section 2.5.

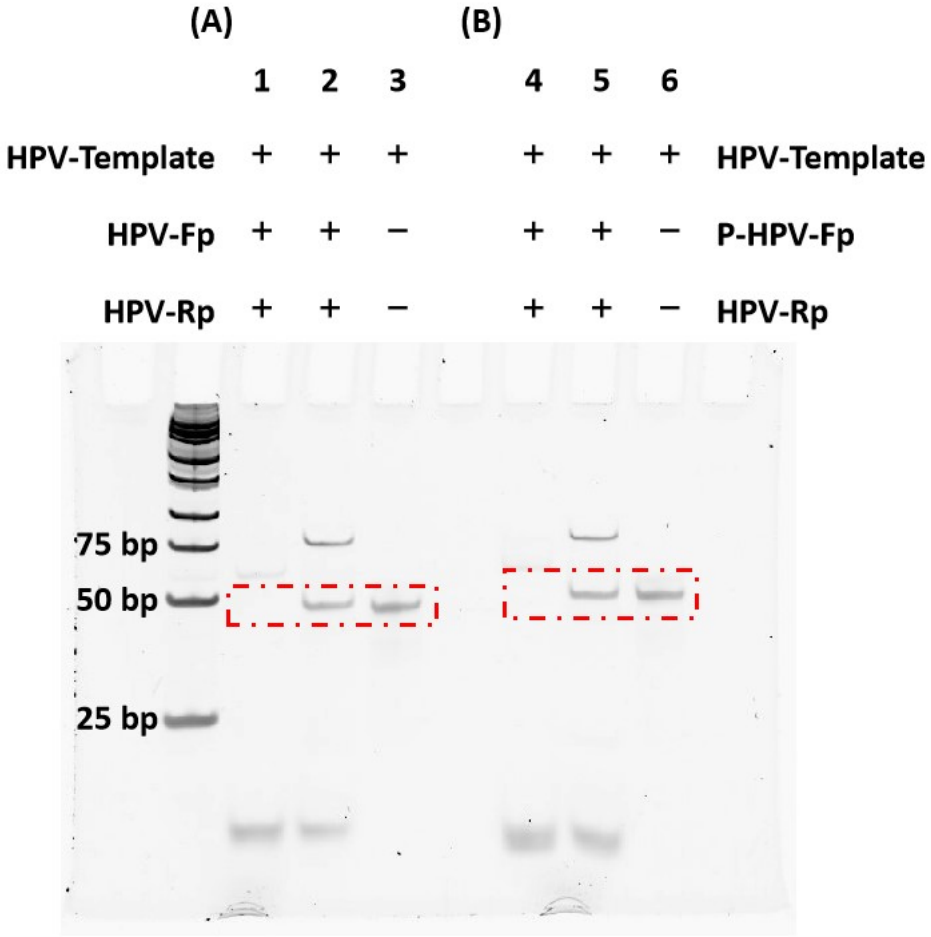

Figure S16. Gel electrophoresis characterization of LATE-PCR products of the HPV gene. (A) NSNAP-III detection system. Lane 1 shows the HPV gene template along with upstream and downstream primers, and Lane 2 shows the amplification product. (B) NSNAP-λ detection system. Lane 4 shows the HPV gene template and primers, and Lane 5 shows the amplification product. The single-stranded target was successfully amplified, with the target strand band highlighted in red. The template strand concentration was 1000 fM. Details of the gel electrophoresis characterization are provided in Experimental Section 2.4, and the LATE-PCR experiment is described in Experimental Section 2.5.

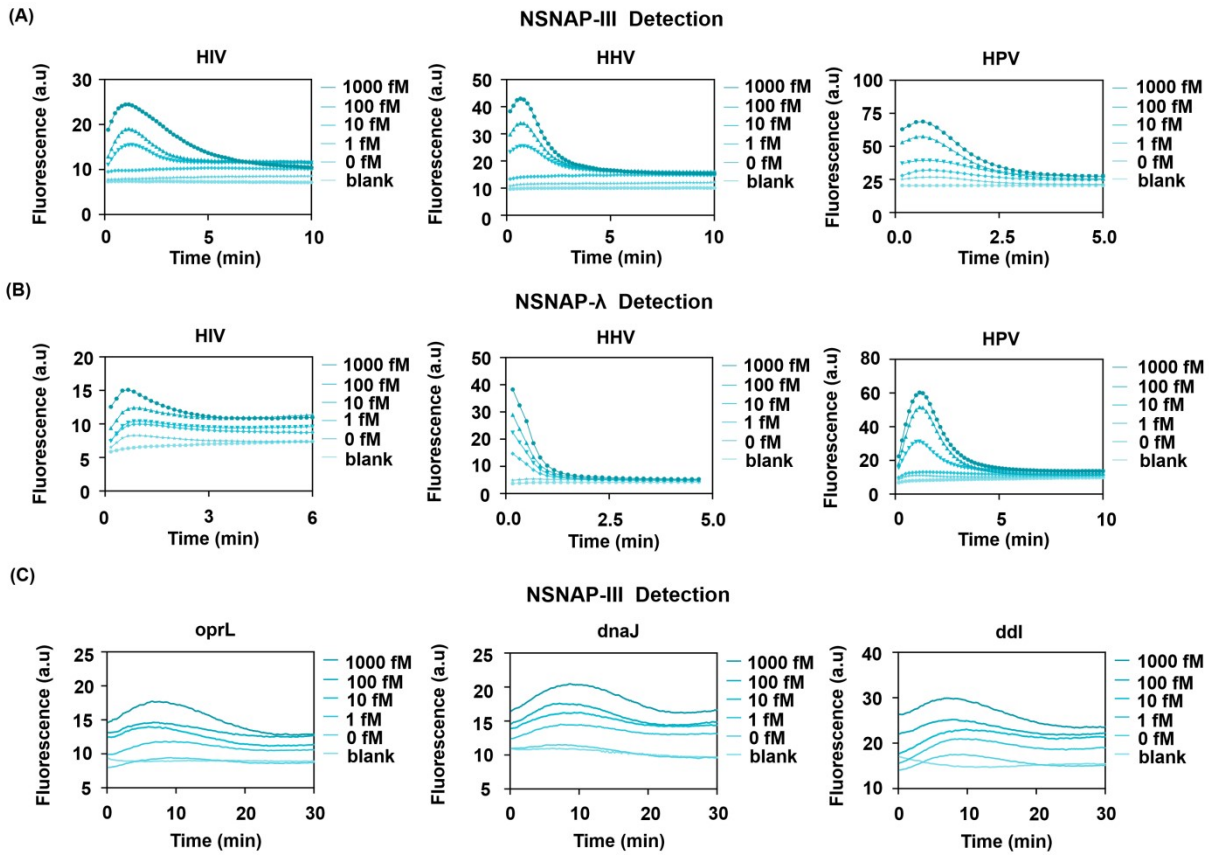

Figure S17. Fluorescence kinetic curves for the quantitative analysis of viral and bacterial genes using NSNAP. (A) Fluorescence kinetic curves for the quantitative analysis of HIV, HHV, and HPV genes using NSNAP-III. (B) Fluorescence kinetic curves for the quantitative analysis of HIV, HHV, and HPV genes using NSNAP-λ. (C) Fluorescence kinetic curves for the quantitative analysis of bacterial genes *oprL, dnaJ*, and *ddl* using NSNAP-III. All reactions used a nucleic acid probe concentration of 50 nM. Exo III and λ Exo were used at concentrations of 250 U/mL and 500 U/mL, respectively. Data are the means ± s.d. (n = 3 independent experiments).

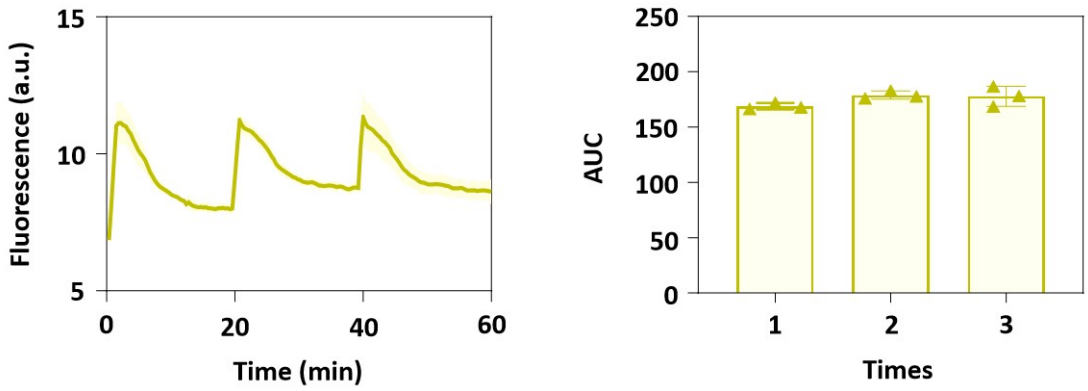

Figure S18. Fluorescence kinetic curves and corresponding area under the curve (AUC) values generated by NSNAP-III for the detection of the bacterial gene *dnaJ* over three consecutive reuse cycles. The nucleic acid probe concentration was 50 nM, and the target (*dnaJ* plasmid) concentration was 100 fM. In all experiments, the Exo III concentration was 250 U/mL, and NEBuffer 1 was used as the reaction buffer. Experiments were conducted at 37 ℃. Data are the means ± s.d. (n = 3 independent experiments).

Supporting Tables

Table S1. Sequence of NSNAP-III system

Name	Sequence (5'-3')
S1	AGTCTCGTCGTTGCGACCAAGCTTCGCTTATGGT AACCTGTGCTCCTTG*C*T*T*C*C
S2	AAGGAGCACAGGTTACC*A*T*A*A*G
S3	CGAAGCTTGGTCGCA*A*C*G*A*C
S4 (Target/Match)	CGAAGCTTGGTCGCAACGACGAGACT
Single mismatch	CGAAGCTTGGTCGCAACGA**A**GAGACT
Multiple mismatch	CGAAGCTTGGTCGCAAC**ACA**GAGACT

Table S2. Sequence of NSNAP-λ system

Name	Sequence (5'-3')
S5	A*T*G*G*T*ACGGGTTAAAGGTGTCTTATGGTAA CCTGTGCTCCTTTCATAACGCCTCCTTCCA
S6	*A*C*A*C*CTTTAACCCGTACCAT
S7	A*A*G*G*A*GCACAGGTTACCATAAC
S8	TGGAAGGAGGCGTTATGAAAGGAGCACAGGTTA

Table S3. Viral gene detection (NSNAP-III)

Species	Name	Sequence (5'-3')
HIV	HIV-Template (Non-target 1)	GGAACCCACTGCTTAAGCCTCAATAAA GCTTGCCTTGAGTGCTTCAAGTAGTGT GTGCCCGTCTGTTGTGTGACTC
	HIV-Fp	GGAACCCACTGCTTAAGCCT
	HIV-Rp	GAGTCACACAACAGACGGGC
	HIV- S1	GAGTCACACAACAGACGGGCACACACT ACTCTTATGGTAACCTGTGC*T*C*C*T*T
	HIV- S3	TAGTGTGTGCCCG*T*C*T*G*T
	S2	AAGGAGCACAGGTTACC*A*T*A*A*G
HPV	HPV-Template (Non-target 2)	GTGTGACTCTACGCTTCGGTTGTGCGT ACAAAGCACACACGTAGACATTCGTAC TTTGGAAGACCTGTTAATGGGC
	HPV-Fp	GTGTGACTCTACGCTTCGGT
	HPV-Rp	GCCCATTAACAGGTCTTCCA
	HPV-S1	GCCCATTAACAGGTCTTCCAAAGTACG AATCTTATGGTAACCTGTGC*T*C*C*T*T
	HPV- S3	TCGTACTTTGGAA*G*A*C*C*T
	S2	AAGGAGCACAGGTTACC*A*T*A*A*G
HHV	HHV-Template (Non-target 3)	ACCAGTTAGAGGGGTCTGCAAAACCCT CTGAGAATACACAGCATTAAAATTCTC GTTCTTCCTCAAATGACCCGAGAGATG ATTTTGCGT
	HHV-Fp	ACCAGTTAGAGGGGTCTGCA
	HHV-Rp	ACGCAAAATCATCTCTCGGGT
	HHV- S1	ACGCAAAATCATCTCTCGGGTCATTTG

		AGGACTTATGGTAACCTGTGC*T*C*C*
		T*T
	HHV- S3	**CTCAAATGACCCGA*G*A*G*A*T**
	S2	**AAGGAGCACAGGTTACC*A*T*A*A*G**

Table S4. Viral gene detection (NSNAP-λ)

Species	Name	Sequence (5'-3')
HIV	**HIV-Template**	**GGAACCCACTGCTTAAGCCTCAATAA**
	(Non-target 1)	**AGCTTGCCTTGAGTGCTTCAAGTAGT**
		GTGTGCCCGTCTGTTGTGTGACTC
	P-HIV-Fp	**GGAACCCACTGCTTAAGCCT**
	HIV-Rp	**GAGTCACACAACAGACGGGC**
	HIV- S5	***A*T*G*G*TACGGGTTAAAGGTGTGC**
		AAGCTTTATTGAGGCTTAAGCAGTGG
		GTTCC
	S6	***A*C*A*C*CTTTAACCCGTACCAT**
	HIV-S7	***A*G*C*C*TCAATAAAGCTTGC**
HPV	**HPV-Template**	**GTGTGACTCTACGCTTCGGTTGTGCG**
	(Non-target 2)	**TACAAAGCACACACGTAGACATTCGT**
		ACTTTGGAAGACCTGTTAATGGGC
	P-HPV-Fp	**GTGTGACTCTACGCTTCGGT**
	HPV-Rp	**GCCCATTAACAGGTCTTCCA**
	HPV-S5	***A*T*G*G*TACGGGTTAAAGGTGTCTT**
		TGTACGCACAACCGAAGCGTAGAGTC
		ACAC
	S6	***A*C*A*C*CTTTAACCCGTACCAT**
	HPV-S7	***T*C*G*G*TTGTGCGTACAAAG**
	HHV-Template	**ACCAGTTAGAGGGGTCTGCAAAACCC**
	(Non-target 3)	**TCTGAGAATACACAGCATTAAAATTCT**
		CGTTCTTCCTCAAATGACCCGAGAGA

		TGATTTTGCGT
HHV	**P-HHV-Fp**	**ACCAGTTAGAGGGGTCTGCA**
	HHV-Rp	**ACGCAAAATCATCTCTCGGGT**
	HHV-S5	***A*T*G*G*TACGGGTTAAAGGTGTTCT**
		CAGAGGGTTTTGCAGACCCCTCTAAC
		TGGT
	S6	***A*C*A*C*CTTTAACCCGTACCAT**
	HHV-S7	***C*T*G*C*AAAACCCTCTGAGA**

Table S5. Bacterial gene detection (NSNAP-III)

Species	Name	Sequence (5'-3')
	oprL-	**ATGGAAATGCTGAAATTCGGCAAATTTGCTGCGCTGG**
	plasmid	**CTCTGGCCATGGCTGTGGCTGTGGGTTGCTCCTCCAA**
		GGGCGGCGATGCTTCCGGTGAAGGTGCCAATGGCGG
		CGTCGACCCGAACGCAGGCTATGGCGCCAACAGCGGT
		GCCGTTGACGGCAGCCTGAGCGACGAAGCCGCTCTGC
		GTGCGATCACCACCTTCTACTTCGAGTACGACAGCTC
		CGACCTGAAGCCGGAAGCCATGCGCGCTCTGGACGTA
		CACGCGAAAGACCTGAAAGGCAGCGGTCAGCGCGTA
		GTGCTGGAAGGCCACACCGACGAACGCGGCACCCGC
oprL		**GAGTACAACATGGCTCTGGGCGAGCGTCGTGCCAAGG**
		CCGTTCAGCGCTACCTGGTGCTGCAGGGCGTTTCGCC
		GGCCACGCTGGAACTGGTTTCCTATGGTAAAGAGCGT
		CCGGTCGCTACCGGCCACGACGAGCAGTCCTGGGCTC
		AGAACCGTCGCGTCGAGCTGAAGAAGTAA
	oprL-Fp	**TACTTCTTCAGCTCGACGCGACG**
	oprL-Rp	**CTACCTGGTGCTGCAGGG**
	oprL- S1	**CTACCTGGTGCTGCAGGGCGTTTCGCCGGCCTTATGG**
		TAACCTGTGC*T*C*C*T*T
	oprL- S3	**GCCGGCGAAACGCCCTGCA*G*C*A*C*C**

	S2	AAGGAGCACAGGTTACC*A*T*A*A*G
	dnaJ-	ATGGATCTGACGCTGGAAGAGGCTGTTCGCGGTGTCA
	plasmid	CCAAAGAGATCCGTATCCCGACGCTGGAAAGTGTGAC
		GTTTGCCATGGCAGCGGTGCGAAAGCGGGTACGCAGC
		CACAAACCTGTCCAACCTGTCACGGTTCCGGTCAGGT
dnaJ		GCAGATGCGTCAGGGCTTTTTCGCCGTTCAGCAGGCA
		TGTCCGCACTGTCATGGTCGTGGGACGCTGATTAAAG
		ATCCATGCACCAAATGCCACGGCCACGGTCGCGTTGA
		GAAAACCAAAACCCTGTCCGTTAAAATCCCGGCTGGC
		GTAGATACGGGTGACCGCATCCGTCTGGCAGGTGAAG
		GCGAAGCAGGCGAGCACGGTGCACCAGCAGGCGATC
		TGTACGTTCAGGTTCAGGTGAAACAGCACGCTATCTT
		TGAGCGTGAAGGCAACAACCTCTACTGTGAAGTCCCG
		ATCAACTTTGCTATGGCAGCGCTCGGTGGCGAAATAG
		AAGTGCCTACGCTGGATGGGCGCGTGAACCTGAAAAT
		CCCAGGCGAAACGCAGACCGGTAAGCTGTTCCGCATG
		CGCGGTAAAGGCGTGAAATCCGTTCGCGGTGGTGCGC
		AGGGCGACCTGCTGTGCCGCGTGGTGGTTGAAACCCC
		GGTTGGCCTGAATGACAAGCAGAAACAGCTGTTAAAA
		GAGCTGCAGGAAAGCTTTGGCGGCCCGACGGGTGAG
		AAAAACAGCCCACGCTCCAAAAGCTTCTTCGATGGCG
		TCAAAAATTCTTCGATGA
	dnaJ-Fp	GGGATTTTCAGGTTCACGCGC
	dnaJ-Rp	GGCAACAACCTCTACTGTGA
	dnaJ-S1	GGCAACAACCTCTACTGTGAAGTCCCGATCCTTATGG
		TAACCTGTGC*T*C*C*T*T
	dnaJ- S3	GATCGGGACTTCACAGTAG*A*G*G*T*T
	S2	AAGGAGCACAGGTTACC*A*T*A*A*G
	ddl-plasmid	CCTTATGTCGGCGCAGGCGTATTGACCAGTGCATGTG

		CCATGGATAAAATCATGACCAAGTATATTTTACAAGCT
		GCTGGTGTGCCGCAAGTTCCTTATGTACCAGTACTTA
		AGAATCAATGGAAAGAAAATCCTAAAAAAGTATTTGA
ddl		TCAATGTGAAGGTTCTTTGCTTTATCCGATGTTTGTCA
		AACCGGCGAATATGGGTTCTAGTGTCGGCATTACAAA
		AGCAGAAAACCGAGAAGAGCTGCAAAATGCTTTAGCA
		ACAGCCTATCAGTATGATTCTCGAGCAATCGTTGAAC
		AAGGAATTGAAGCGCGCGAAATCGAAGTTGCTGTATT
		AGGAAATGAAGACGTTCGGACGACTTTGCCTGGTGAA
		GTCGTAAAAGACGTAGCATTCTATGATTATGAAGCAA
		AATATATCAATAATAAAATCGAAATGCAGATTCCAGCC
		GAAGTGCCAGAAGAAGTTTATCAAAAAGCGCAAGAGT
		ACGCGAAGTTAGCTTACACGATGTTAGGTGGAAGCGG
		ATTGAGCCGGTGCGATTTCTTTTTGACAAATAAAAATG
		AATTATTCCTGAATGAATTAA
	ddl-Fp	ACTTCGGCTGGAATCTGCATTTC
	ddl-Rp	GAAGACGTTCGGACGACT
	ddl- S1	GAAGACGTTCGGACGACTTTGCCTGGTGAACTTATGG
		TAACCTGTGC*T*C*C*T*T
	ddl- S3	TTCACCAGGCAAAGTCGTC*C*G*A*A*C
	S2	AAGGAGCACAGGTTACC*A*T*A*A*G

Note:
*Indicates the base is modified with phosphorothioates. The 5' end of the upstream primer for the viral gene in the NSNAP-λ detection system is phosphorylated.
The nucleic acid probe in the NSNAP-III system consists of three strands: S1, S2, and S3. The nucleic acid probe in the NSNAP-λ system consists of three strands: S5, S6, and S7.

Table S6. Comparative Performance Metrics of NSNAP and Established Nucleic Acid Detection Technologies

Parameter	NSNAP	Traditional	Digital PCR	CRISPR-based

		Probes		Assays
Sensitivity	**~1 fM**	**~10-100 fM**	**~1 fM**	**~1-10 fM**
Time-to-result	**2-3 hours**	**1.5-2 hours**	**3-5 hours**	**3-4 hours**
Reusability	**Up to 7 cycles**	**Single-use**	**Single-use**	**Single-use**
Instrumentation	**Standard qPCR**	**Standard qPCR**	**Specialized (droplet)**	**Basic + Cas handling setup**
Approximate Cost	**Low (due to reuse)**	**Medium-low**	**High**	**Medium-high**