Electronic Supplementary Information

An ultrasensitive terminal protection-based real-time fluorescence approach for protein detection via isothermal exponential amplification

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1. Synthesized oligonucleotide sequences uesed in the experiments.

Table S1. Synthesized oligonucleotide sequences uesed in the experiments.

Oligonucleotide	Sequences (5'~ 3')
Template X'-Y'-Biotin	CCTACGACTG-TCCAGACTCT-ATGGTAGTTCGGCGTC-TTTT-Biotin
Template X'-Y'-Folate	CCTACGACTG-TCCAGACTCT-ATGGTAGTTCGGCGTC-TTTT-Folate
Template X'-X'	CCTACGACTG-AACAGACTCT-CCTACGACTG-P
Primer Y	GACGCCGACTACCAT

2. The fluorescence curves caused by the reactions of DNA-linked and free small molecules with target protein respectively.

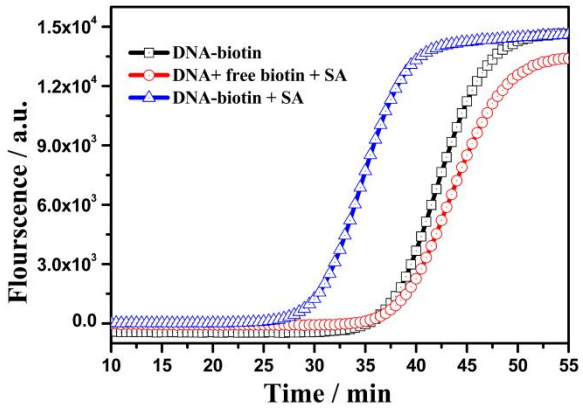

Fig. S1. The fluorescence curves caused by the reactions of DNA-linked and free small molecules with target protein respectively. The DNA-biotin is the Template X'-Y'-Biotin. The DNA is the Template X'-Y' without biotin labeled at its 3-terminal. The DNA-biotin, DNA, and free biotin is 1 nM respectively. The SA is 10 pM. Other experiment conditions are the same as those described in the experimental section. Final concentrations: [Exo I] = 20 U, [primer Y] = 0.1 nM, [template X'-X'] = 0.1 μM, [each dNTP] = 250 μM, [Nt.BstNBI] = 0.4 U/μL, [Vent (exo-) DNA polymerase] = 0.02 U/μL, and [SG] = 0.4 μg/mL.

To further confirm that the fluorescence signal is produced by specific binding between protein and small molecule labeled on DNA, we have compared the real-time fluorescence curves of DNA-biotin and free biotin reacting with SA. As Fig. S1 shown, in the presence of the free biotin and DNA, the target protein-SA produces a fluorescence signal similar to that produced by DNA-biotin without SA (blank). Their POI values are larger. Only the interaction of SA with DNA-binotin can produce the smaller POI value. This suggests that the effective fluorescence signal comes from the specific interaction of SA with biotin labeled on DNA. The interaction of SA with the free biotin does not

produce an effective fluorescence signal. This experiment also fully proves the feasibility of our proposed principle.

3. Optimization of the dosage of Exo I on the protein detection.

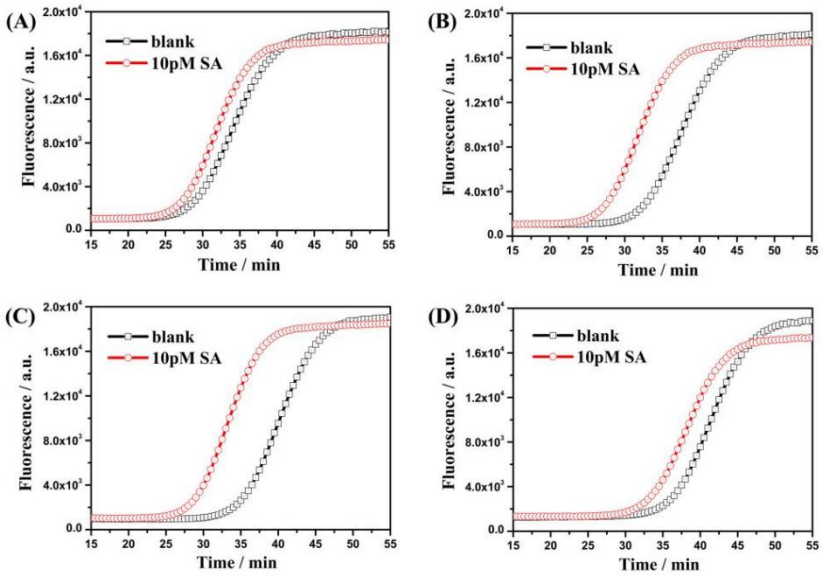

Fig. S2. The effect of the dosage of Exo I on protein detection. The real-time fluorescence curves are produced by 10 pM SA and the blank. The blank is treated in the same way without SA. The amount of Exo I from A to D is 5, 10, 20, and 30 U, respectively. Other experiment conditions are the same as those described in the experimental section. Final concentrations: [primer Y] = 0.1 nM, [template X'-Y'-biotin] = 1 nM, [template X'-X'] = 0.1 μM, [each dNTP] = 250 μM, [Nt.BstNBI] = 0.4 U/μL, [Vent (exo-) DNA polymerase] = 0.02 U/μL, and [SG] = 0.4 μg/mL.

Exo I is used to degrade the template X'-Y'-biotin which is not protected by proteins. Especially in the blank solution, if the degradation of template X'-Y'-biotin isn't complete, it will directly lead to the growth of the blank fluorescence signal. To investigate the influence of the concentration of Exo I on SA detection, we select various concentrations of Exo I from 5 to 30 U to conduct the experiments with 10 pmol SA sample. As shown in Fig. S2, with the increase of the dosage of Exo I, the POI value gradually increases. When the amount of Exo I is changed from 20 U to 30 U, the POI values of the blanks hardly change. It indicates that the 20 U Exo I has wholly degraded the template X'-Y'-biotin in the blank. Moreover, when the Exo I dosage is 20 U, the interval of the POI between SA and the blank is the largest. Therefore, 20 U Exo I is used as the optimum amount in protein detection.

4. Optimization of the degradation time of Exo I on protein detection.

The degradation time of Exo I has an essential influence on the degradation efficiency of template X'-Y'-biotin. The influence of the degradation time of Exo I on SA detection is investigated. As shown in Fig. S3, when the degradation time of Exo I is 30 min, the interval of the POI between SA and

blank is the largest. Therefore, 30 min is considered the optimum degradation time of Exo I in protein detection.

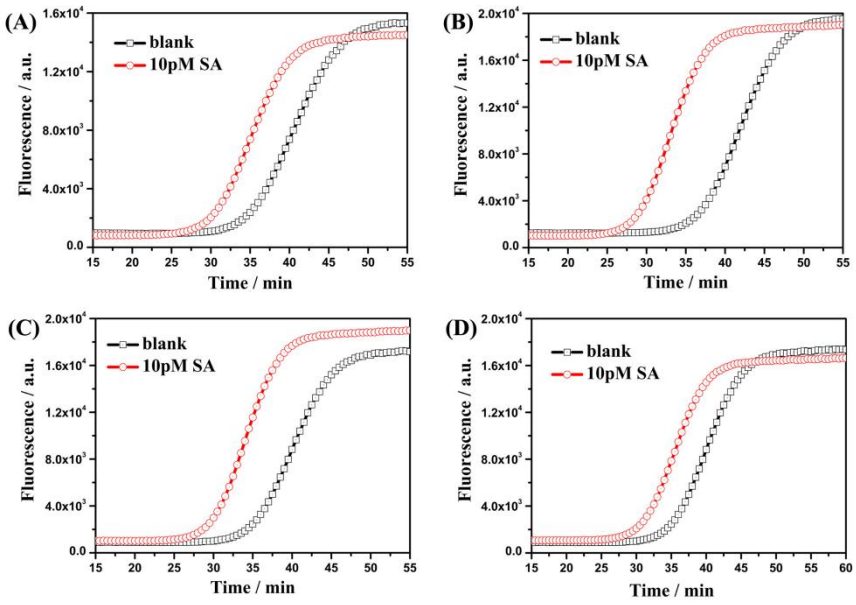

Fig. S3. The effect of the digestion time of Exo I on protein detection. The real-time fluorescence curves are produced by 10 pM SA and blank. The blank is treated in the same way without SA. The digestion time of Exo I is 15 min (A), 30 min (B), 45 min (C), and 60 min (D), respectively. Other experiment conditions are the same as those described in the experimental section. Final concentrations: [Exo I] = 20 U, [primer Y] = 0.1 nM, [template X'-Y'-Biotin] = 1 nM, [template X'-X'] = 0.1 μM, [each dNTP] = 250 μM, [Vent (exo-) DNA polymerase] = 0.02 U/μL, [Nt.BstNBI] = 0.4 U/μL, and [SG] = 0.4 μg/mL.

5. The effect of the concentration of Vent (exo-) DNA polymerase on protein detection.

Vent (exo$^-$) DNA Polymerase is an important reactant of IEXPAR. Its dosage can directly affect the amplification efficiency of IEXPAR. We have investigated the effect of the dosage of Vent (exo$^-$) DNA polymerase on SA detection. As shown in Fig. S4, accompanying the increase of polymerase dosage, the POI values of sample and blank are getting smaller. That's because the larger the dosage of polymerase, the faster the nucleic acid extends in the SA sample. At the same time, the earlier the non-specific amplification occurs in the blank. When the concentration of Vent (exo-) DNA polymerase is 0.02 U/μL, the interval of the POI between SA and blank is the largest. Therefore, 0.02 U/μL is used as the optimum dosage of Vent (exo$^-$) DNA polymerase in protein detection.

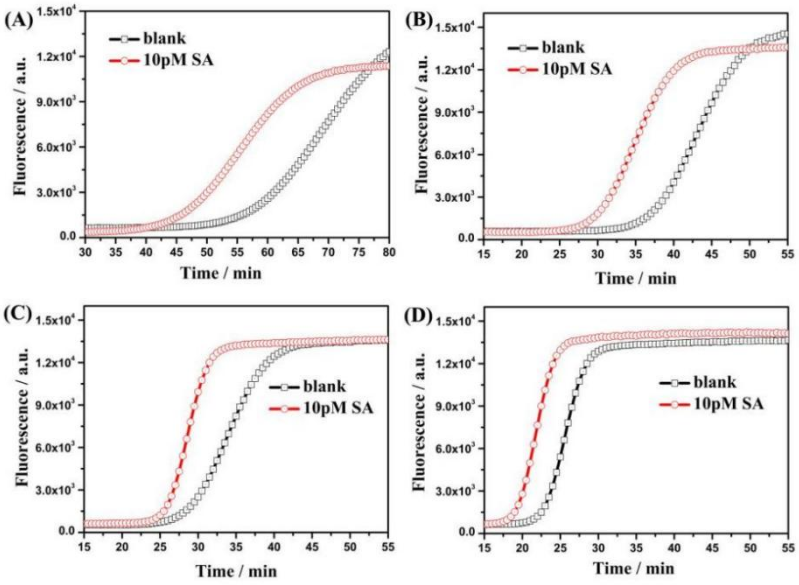

Fig. S4. The effect of the concentration of Vent (exo-) DNA polymerase on the protein detection. The real-time fluorescence curves produced by 10 pM SA are compared in the presence of 0.01, 0.02, 0.03, and 0.04 U/μL Vent (exo-) DNA polymerase, respectively. The blank is treated in the same way without SA. Other experiment conditions are the same as those described in the experimental section. Final concentrations: [Exo I] = 20 U, [primer Y] = 0.1 nM, [template X'-Y'-Biotin] = 1 nM, [template X'-X'] = 0.1 μM, [each dNTP] = 250 μM, [Nt.BstNBI] = 0.4 U/μL, and [SG] = 0.4 μg/mL.

6. The effect of the concentration of Nt.BstNBI nicking endonuclease on protein detection.

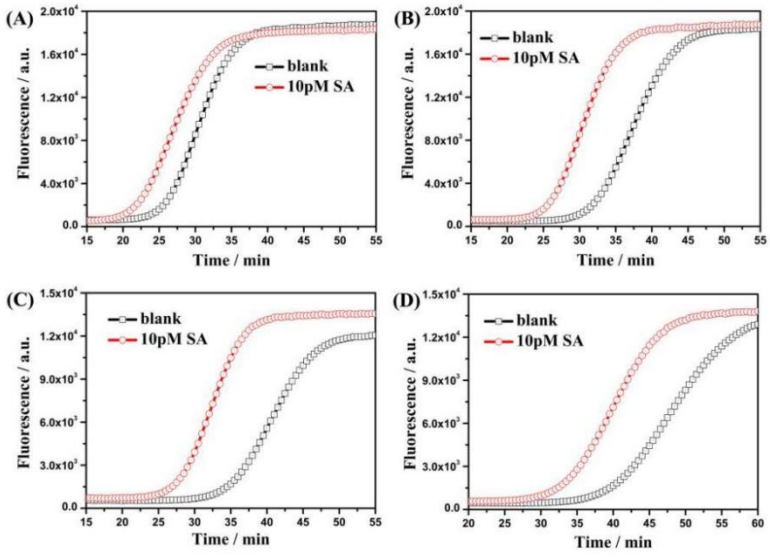

Fig. S5. The effect of the dosage of Nt.BstNBI nicking endonuclease. The real-time fluorescence curves are produced by 10 pM SA and the blank. The blank is treated in the same way without SA. The concentration of Nt.BstNBI nicking endonuclease is 0.2, 0.3, 0.4, and 0.5 U/μL, respectively. Other experiment conditions are the same as those described in the experimental section. Final concentrations: [Exo I] = 20 U, [primer Y] = 0.1 nM, [template X'-Y'-Biotin] = 1 nM, [template X'-X'] = 0.1 μM, [each dNTP] = 250 μM, [Vent (exo-) DNA polymerase] = 0.02 U/μL, and [SG] = 0.4 μg/mL.

We investigate the influence of the concentration of Nt.BstNBI nicking endonuclease from 0.2 to 0.5 U/μL on the SA detection (Fig. S5). As the concentration of nicking endonuclease increases, the POI values of the fluorescence curves produced by the SA sample and blank increase gradually. We speculate the reason. The IEXPAR is accomplished by the joint action of polymerase and nicking endonuclease. When the concentration of nicking endonuclease is small (0.2 U/μL, Fig. S5A), the polymerase is relatively excessive. Excess polymerase results in rapid exponential amplification in the sample and severe non-specific amplification in the blank. Therefore, the POI values of the blank and the sample are relatively small. With the increase of nicking endonuclease concentration, the degree of endonuclease cleavage and consumption of polymerase increase gradually in the linear amplification stage. The amount of polymerase left for the exponential amplification gradually decreases. The sample needs more time to amplify to achieve exponential signal growth. Therefore, the POI of sample gradually increases. At the same time, the decrease of polymerase weakens the non-specific amplification of blank, and the POI of blank is gradually increased. When the concentration of Nt.BstNBI is 0.4 U/μL, the interval of the POI between SA and blank is the largest (Fig. S5C). Therefore, 0.4 U/μL is used as the optimum dosage of Nt.BstNBI nicking endonuclease in protein detection.

7. The effect of the concentration of template X'-X' on protein detection.

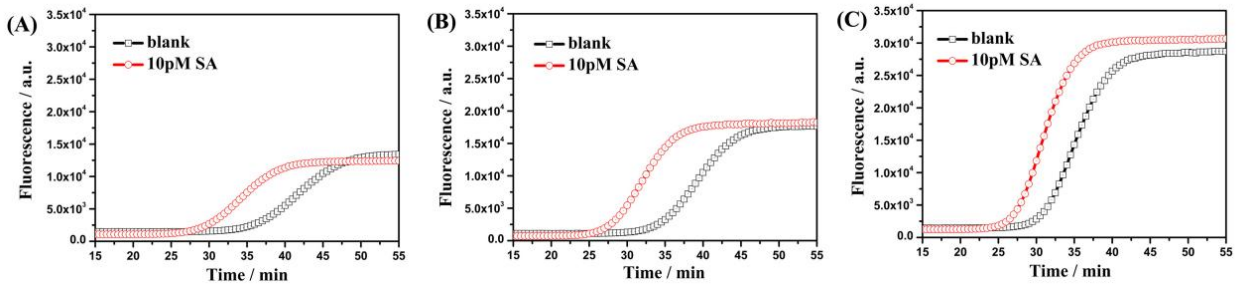

Fig. S6. The influence of the concentration of template X'-X'. The real-time fluorescence curves are produced by 10 pM SA. The concentrations of template X'-X' are 0.05, 0.1, and 0.2 μM, respectively. The blank is treated in the same way without SA. Other experiment conditions are the same as those in described the experimental section. Final concentrations: [primer Y] = 0.1 nM, [template X'-Y'-Biotin] = 1 nM, [Exo I] = 20 U, [each dNTP] = 250 μM, [Vent (exo-) DNA polymerase] = 0.02 U/μL, [Nt.BstNBI] = 0.4 U/μL, and [SG] = 0.4 μg/mL.

The amount of double-stranded DNA (dsDNA) produced by the IEXPAR amplification reaction depends on the dosage of the template X'-X'. So the concentration of the template X'-X' has an important influence on the intensity of the real-time fluorescence signal. As shown in Fig. S6, with the increase of template X'-X' concentration, the fluorescence signal intensities caused by SA and blank become stronger after amplification. When the concentration of template X'-X' is 0.1 μM, the interval

of the POI between SA and blank is the largest. Therefore, 0.1 μM is considered the optimum concentration of template X'-X' in the protein detection.

8. Estimation of the influence of interfering proteins on the detection of target proteins.

First, the POI values of the real-time fluorescence curves produced by SA, thrombin, HAS, IgG, and FR are defined as POI_a, POI_b, POI_c, POI_d, and POI_e, respectively. According to the dynamic linear equation (Fig. 2B, POI = -7.02 - 3.47 lgC_{SA}), the concentrations of SA corresponding to the POI signals generated by the interference proteins are calculated as C_a, C_b, C_c, C_d, and C_e. According to the above equation, the following equations can be obtained.

$$POI_b - POI_a = -3.47\,(lgC_b - lgC_a) \quad (1)$$

$$lg\frac{C_b}{C_a} = -\frac{POI_b - POI_a}{3.47} \quad (2)$$

According to equation (2) and the POI values of each protein, we can calculate the relative concentration of each interfering protein relative to the target protein SA. As shown in Fig. 3A, the POI values of SA, thrombin, HAS, IgG, and FR are 32.5, 39, 41, 38, and 37.5, respectively. The calculated relative concentrations of C_b/C_a, C_c/C_a, C_d/C_a, and C_e/C_a are 1.35%, 0.35%, 2.60%, and 3.62%, respectively. The relative concentration can represent the influence of interfering proteins on the detection of target protein.

In the selective analysis of FR, we used the same estimation method to calculate the effect of interfering proteins on FR detection according to the dynamic linear equation of Fig. 4B (POI = 20.59 - 2.00 lgC_{FR}). As shown in Fig. 5A, the POI values of FR, IgG, thrombin, HSA, and SA are 35, 40, 41.5, 39.5, and 38.5, respectively. The calculated their relative concentrations are 0.32%, 0.06%, 0.56%, and 1.78%, respectively.

9. Comparison of various methods for protein assay.

Table S2 Comparison of various methods for protein assay.

Method	Amplification	Detection signal	The lowest detection concentration	Reference
Terminal protection	None	Fluorescence	5 nM SA	[11]
	None	Fluorescence	0.1 nM SA	[12]
	RCA	Electrochemistry	1 pM SA	[15]
	Exo III-assisted amplification	Fluorescence	69 pM SA	[16]
	Exo I-assisted amplification	Fluorescence	20 pM SA	[17]
	Cascade signal amplification	Fluorescence	1 pM FR (0.44	[14]
	DNAzyme amplification	Electrochemistry	1 ng/mL FR	[19]
	Cascade signal amplification	Colorimetry	1 ng/mL FR	[20]
	Exo I-assisted amplification	Fluorescence	0.2 ng/mL FR	[21]
	IEXPAR	Real-time fluorescence	100 fM SA; 0.2 ng/mL FR	Our method
Aptamer	IEXPAR	Real-time fluorescence	100 fM thrombin	[S1]
	IEXPAR	Real-time fluorescence	10 fM PDGF-BB*	[S2]
Immunoassay	IEXPAR	Colorimetry	0.1 ng/L PDGF-BB*	[S3]
	IEXPAR	Real-time fluorescence	3 pM MUC1**	[S4]

*PDGF-BB: plate-derived growth factor BB.

**MUC1: tumor protein Mucin 1.

Reference

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