Supporting Information

A label-free double-amplification system for sensitive detection of single-stranded DNA and thrombin by liquid chromatography-mass spectrometry

Wenbo Zhao, Zhen Qin, Chengsen Zhang, Meiping Zhao* and Hai Luo*

Beijing National Laboratory for Molecular Sciences, MOE Key Laboratory of Bioorganic Chemistry and Molecular Engineering, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871 China. Fax: (+86)10-62751708; E-mail: mpzhao@pku.edu.cn; hluo@pku.edu.cn

Tel: +86-10-62758153 Fax: +86-10-62751708

Materials and methods

Chemicals

All the oligonucleotides were purchased from Beijing AuGCT DNA-SYN Biotechnology Co. Ltd (Beijing, China). Exonuclease III (Exo III) along with the reaction buffer, 10 × NEBuffer 1 (100 mM bis-tris-propane-HCl, 100 mM $MgCl_2$ and 10.0 mM dithiothreitol, pH 7.0) and 10 × NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol pH 7.9) was purchased from New England Bio-labs Inc. (Ipswich, MA, USA). Thrombin (> 2000 $U \cdot mg^{-1}$), dAMP, insulin (≥ 27 USP $U \cdot mg^{-1}$), myoglobin (95-100%), lysozyme (≥ 90%) and cytochrome C (≥ 95%) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Acros Organics (Geel, Belgium). HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA).

Detection of the target ssDNA

The ternary DNA probe was prepared by mixing 20 nM Probe-I, 200 nM Probe-II and 20 nM Probe-III and incubating in NEBuffer 1 with the addition of 0.1 M NaCl at 37°C for 10 min. To the obtained solution, the target ssDNA at different concentrations were added and incubated under 37°C for 10 min. Then Exo III was added at a final concentration of 0.2 $U \cdot \mu L^{-1}$ and the mixture was incubated at 37°C for 15 min. After that, the enzyme was deactivated at 72°C for 10 min. The resultant solution was centrifuged at 10000 rpm at room temperature for 30 s and the supernatant was collected and subjected to LC/MS analysis.

Detection of thrombin

The ternary DNA probe was prepared by mixing 20 nM Probe-I, 200 nM Probe-II and 20 nM Probe-III and incubating in NEBuffer 3 at 37°C for 10 min. Different concentrations of thrombin (from 0 to 20 nM) were added to 20 nM AP-strand solution and incubated at 37°C for 10 min [1]. The obtained solution was mixed with the ternary DNA probe solution, to which Exo III was added immediately at a final concentration of 0.2 $U \cdot \mu L^{-1}$. The reaction was carried out at 37°C for 5 min. Then the enzyme was deactivated and the obtained solution was treated in the same way as above. For the specificity test of the assay, the concentrations of all the tested proteins, including thrombin, myoglobin, lysozyme, cytochrome C and insulin, were 20 nM.

HPLC/MS detection

The quantification of dAMP was performed on an Agilent 1200 HPLC coupled to a 6510 ESI-Q-TOF MS using a C18 reversed-phase column (Agilent, ZORBAX Eclipse C18, 150 × 2.1 mm, 5 μm). Chromatograhpic separation was performed by slight modification of previous reported methods.[S1] The column temperature was set at 23°C. The sample injection volume and the flow rate of the mobile phase were 10 μL and 0.1 $mL \cdot min^{-1}$,

respectively. The mobile phase A was 5% methanol aqueous solution (v:v) containing 10.0 mM ammonium acetate (pH 7.0), and the mobile phase B was pure methanol. During the first 5 min, only mobile phase A was run to remove the salt in the sample. Then, the retained analyte dAMP was eluted by mobile phase B and detected by ESI-Q-TOF MS in the negative ion mode with the scan range of *m/z* 150 - 700. The optimized ion source parameters were: nebulizer pressure 40 psi, temperature of drying gas 350°C, flow rate of drying gas 8.0 $L \cdot min^{-1}$, high voltage of the ion source 3.5 kV, and the voltage of fragmentor 150 V, respectively. All the experiments were carried out in triplicate.

The HPLC/MS data were processed by using the Mass Hunter Workstation (Edition B.02.00, Agilent Technologies). The signal of dAMP (*m/z* 330.05 - 330.07) was extracted from the total ion chromatogram (TIC). The integrated peak areas were used for the quantification.

Native polyacrylamide gel electrophoresis (PAGE) analysis

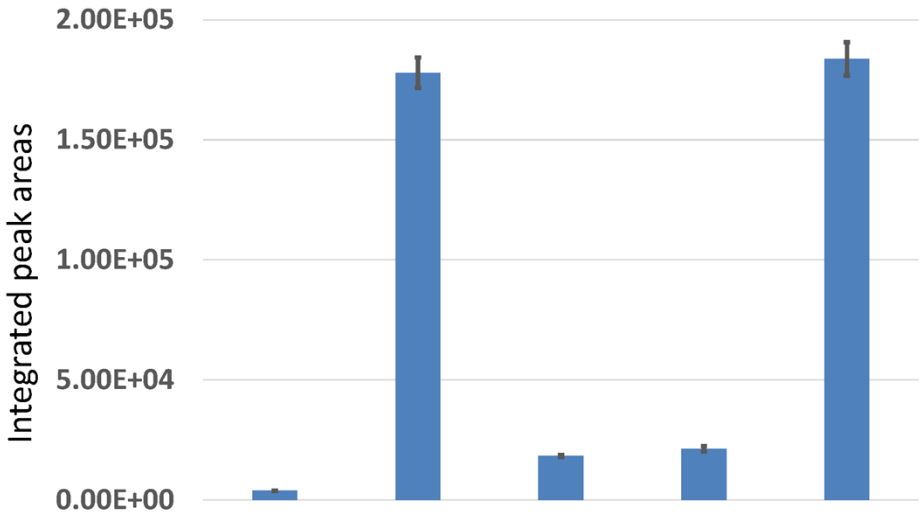
Native PAGE was carried out using 20% (w/v) gel at 15V/cm in a 1×TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA; pH 8.0) for 3 h. Samples (5 μL) with different compositions were loaded into the gel. After separation, the gels containing DNA were stained using GelSafe Dye (Yuanpinghao Bio., Beijing, China), and visualized using a Tanon 1600 gel imaging system (Tanon, China).

Supplementary Results and Discussions

Optimization of the composition of the ternary DNA probe solution

By fixing the concentration of Probe II at 200 nM, we compared the dAMP signal responses at different concentrations of Probe I, Probe III and the target sequence. As shown in Figure S1, the dAMP signal of the fifth column is almost the same as the second column, indicating that Probe I has been efficiently recycled and the amplification reaction

is quite complete. Actually higher concentrations of Probe I and Probe III lead to notable increase of the background signals by comparison between the signals of the fourth column and the first column. From the data shown in the third column, target sequences at a low concentration level (2 nM) only consumed a small amount of Probe II when the reaction was stopped, indicating that 200 nM of Probe II was sufficient for amplification of target sequences at normal concentration levels in biological samples.



Probe-I (nM)	20	20	20	200	200
Probe-II (nM)	200	200	200	200	200
Probe-III(nM)	20	20	20	200	200
Target (nM)	0	20	2	0	20

Figure S1. Comparison of the dAMP signals obtained with different compositions of the ternary DNA probe solution.

The effect of $(AC)_5$-tail in Probe-II

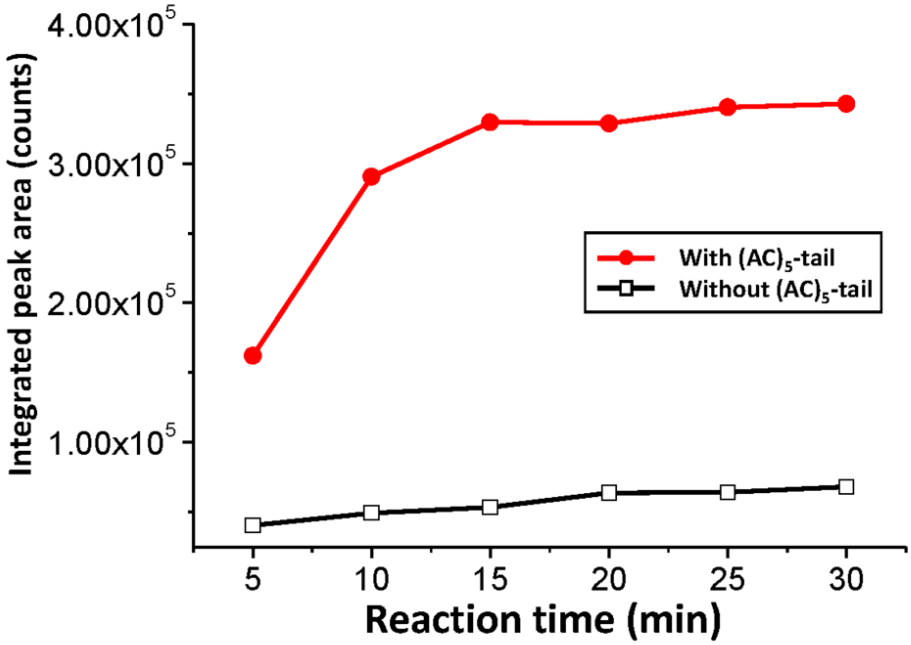


Figure S2. Time curves of dAMP signals obtained by using Probe-II with/without the $(AC)_5$-tail for the amplification reaction.

Original data for Figure 1A

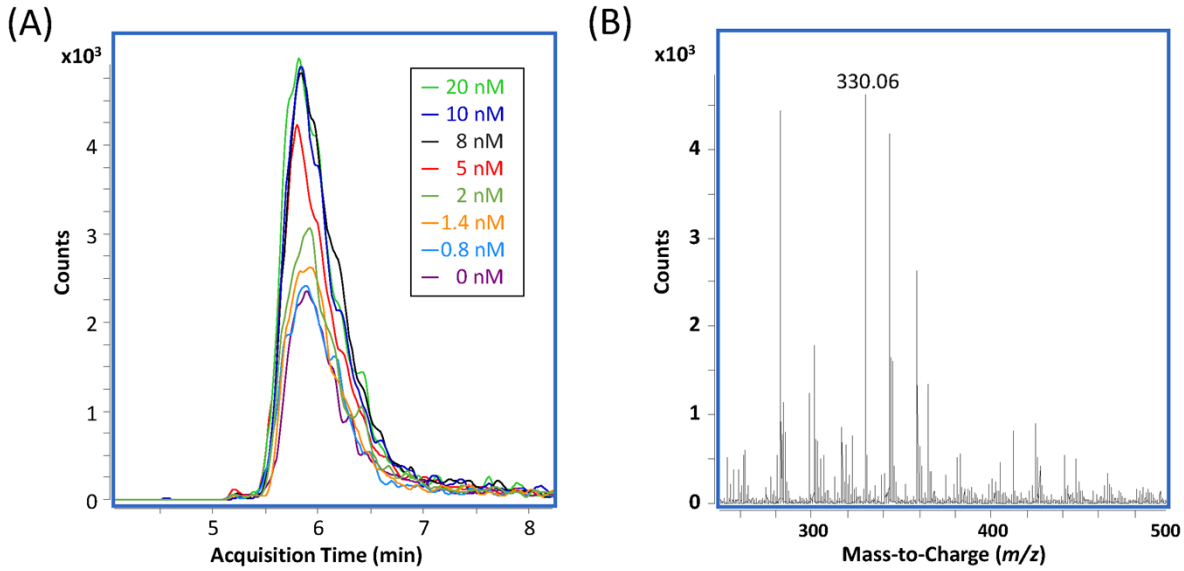


Figure S3. (A) The elected ion chromatograms (EIC) of *m/z* 330.06 (dAMP) at different concentration of target ssDNA. (B) The MS spectrum of the EIC peak in the detection of 60 nM target ssDNA.

Original data for Figure 1B

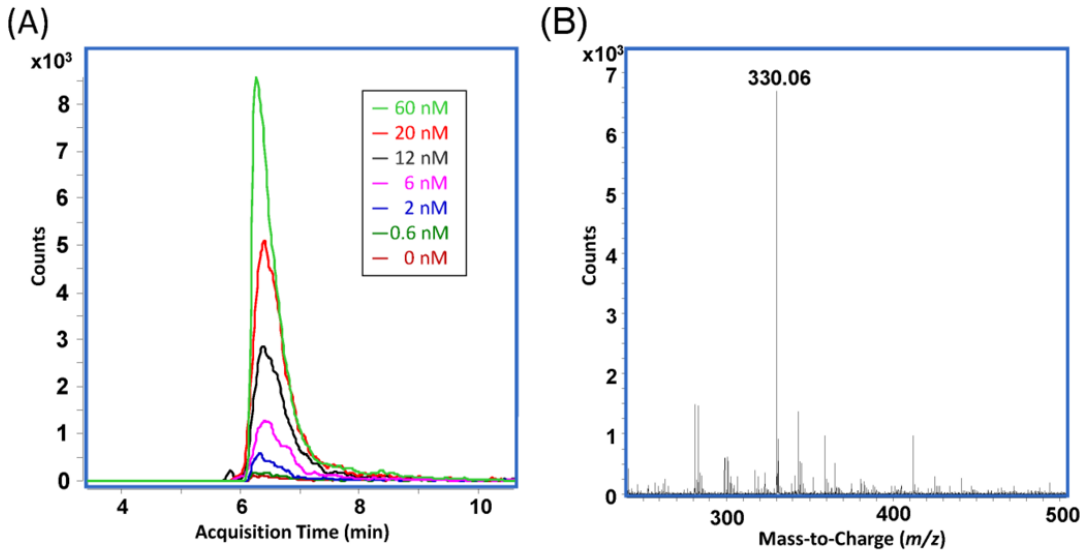


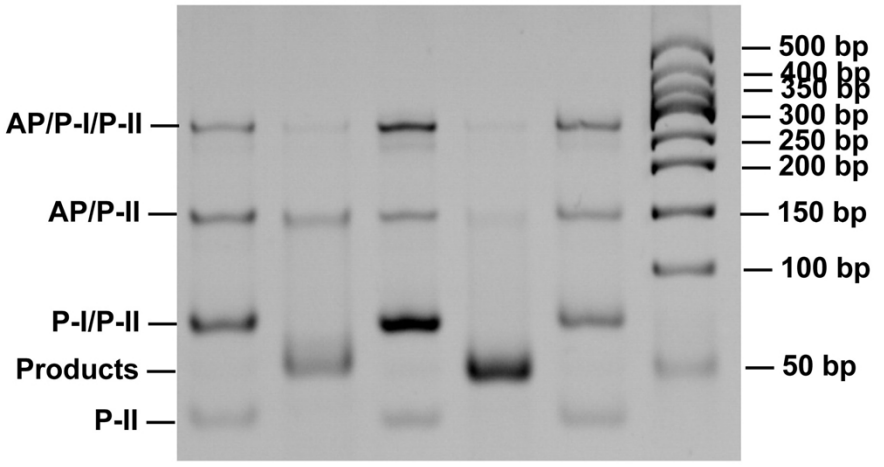
Figure S4. (A) The elected ion chromatograph (EIC) of *m/z* 330.06 (dAMP) at different concentration of thrombin. (B) The MS spectrum of the EIC peak in the detection of 20 nM thrombin.

Possible mechanism of the Exo III-assisted ternary DNA probe double amplification system for the detection of ssDNA and thrombin

To elucidate the possible working mechanism for the amplification reaction, we performed native-PAGE analysis of reaction solutions with different compositions. As shown in Lane 1 of Figure 2A, the mixture solution of Probe-I and Probe-II at a ratio of 1:5 shows two bands in the gel, indicating the presence of Probe-I/Probe-II hybrid and the excess free Probe-II in the solution. Similarly, in Lane 3, the mixture solution of AP strand and Probe-II at a ratio of 1:5 also shows two bands in the gel, corresponding to the hybrid of AP/Probe-II and the excess free Probe-II in the solution, respectively. Lane 6 shows the separation results of a mixture solution of Probe-I, Probe-II and AP-strand at a ratio of 1:5:1. By comparison with the bands in Lane 1 and Lane 3, it can be concluded that the top band in Lane 6 is the desired complex of AP /Probe-I/Probe-II, and the second band from

the top is the hybrid of AP/Probe-II.

Above experiments were all performed in NEBuffer 3 for the detection of thrombin, in which AP strand tends to form a G-quadruplex structure. The results suggested that only a small part of AP strands formed the expected AP/Probe-I/Probe-II complex, while a notable amount of AP strands just bound to the excess free Probe-II and formed AP/Probe-II hybrids in the mixture solution. For confirmation, we further carried out another PAGE analysis by mixing Probe-I, Probe-II and AP in NEBuffer 1, in which the target ssDNA was measured using the amplification reaction. The samples prepared in NEBuffer 3 were also measured in the same experiment for comparison. The results are shown in Figure S5.



	1	2	3	4	5	Marker
NEBuffer	3	3	1	1	1	
P-I (μM)	0.5	0.5	0.5	0.5	0.5	
P-II (μM)	2	2	2	2	2	
AP (μM)	0.5	0.5	0.5	0.5	0.5	
Incubation time (min)	10	10	10	10	0	
Exo III (0.05 U)		√		√		
Reaction (min)		5		5		

Figure S5. Comparison of the native PAGE analysis results of the amplification reaction in different buffer solutions.

From the results shown in Figure S5, in NEBuffer 1, most AP strands formed the expected AP/Probe-I/Probe-II complex, while only a small amount of them formed AP/Probe-II hybrid. Lane 4 in Figure S5 shows that after 5 min, the bands of AP/Probe-I/Probe-II complex, AP/Probe-II hybrid and Probe-I/Probe-II hybrid have all disappeared, indicating that the amplification reaction is generally complete. By comparison the bands in Lane 4 with those in Lane 2 in Figure S5, it can also be seen that the digestion reaction by Exo III in NEBuffer 1 were much faster than that in NEBuffer 3.

By comparison the bands in Lane 5 with those in Lane 1 in Figure 2A, addition of Probe-III seems to have limited influence on the position of the hybrid of Probe-I/Probe-II. Nevertheless, band smearing of the Probe-I/Probe-II hybrid can be clearly seen in Lane 5 in comparison to that in Lane 2, indicating the weak interactions between Probe-III and the Probe-I/Probe-II hybrid under the tested conditions. Theoretically, when Probe-II in the AP/Probe-I/Probe-II complex or AP/Probe-II hybrid is digested to a shorter sequence, the AP-strand tend to leave the shorter hybrid and bind to another intact Probe-I/Probe-II hybrid or intact free Probe-II to form a more stable complex. In this case, Probe-III can take the place of AP-strand and allow Probe-II to be continously digested by Exo III. To confirm this effect of Probe-III, we further carried out a comparison experiments by performing the amplification reaction in the absence and presence of Probe-III. From the results shown in Figure S6, presence of Probe-III in the system indeed greatly accelerated the amplification reaction.

For the thrombin detection system, Lane 10 in Figure 2A shows that addition of thrombin to the AP-strand solution did not show any visible bands. With the addition of thrombin to the mixture solution of AP-strand, Probe-I, Probe-II and Probe-III, the band of AP/Probe-II hybrid in Lane 11 turned darker than that in Lane 7. By contrast, the AP-strand/Probe-I/Probe-II complex became even weaker than that in Lane 7, suggesting that presence of thrombin in the system was beneficial to form the AP/Probe-II hybrid.

In combination with the results shown in Lanes 1 and 2 in Figure S5, we inferred that in

NEBuffer 3, the digestion of Probe-II was mainly initiated by forming the AP/Probe-II hybrid instead of the AP/Probe-I/Probe-II complex. When the first 6-9 nucleotides at the 3' end of Probe-II was removed, the shortened Probe-II may be replaced by another intact Probe-II. The resultant Probe-II fragment may subsequently form the Probe-I/Probe-II/Probe-III complex so that the digestion reaction can continue until all the dAMPs in Probe-II were released. Without Probe-III, the degradation reaction will be much slower. In the presence of thrombin, it tends to bind to the AP strand. With the digestion of Probe-II in the AP/Probe-II hybrid, the shortened Probe-II will dissociate from the AP strand more quickly due to the influence of thrombin, which may facilitate the formation of the Probe-I/Probe-II/Probe-III complex, resulting in acceleration of the increase of dAMP signals. By comparison the bands shown in Lane 12 and Lane 13 with those in Lane 8 and Lane 9 in Figure 2A, presence of thrombin in the system indeed enhanced the reaction and the products were more completely digested after the same reaction time.

Effect of Probe-III on the amplification reaction

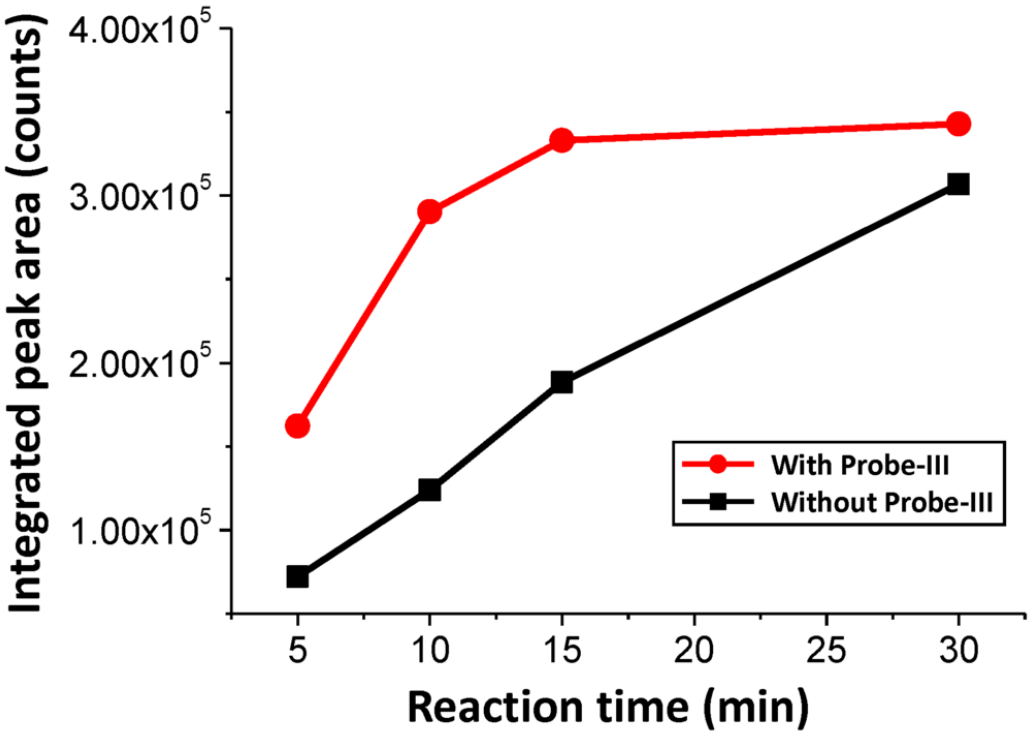


Figure S6. Comparison of the time curves of dAMP signals obtained in the absence or presence of Probe-III in the detection of target ssDNA. The concentration of Probe-I, Probe-II, Probe-III and ExoIII in the experiments were 20 nM, 200 nM, 20 nM and 0.2 $U \cdot \mu L^{-1}$, respectively.

References:

S1. D. M. Tasset, M. F. Kubik and W. Steiner, *Journal of Molecular Biology*, 1997, **272**, 688-698.