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## COMMUNICATION

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

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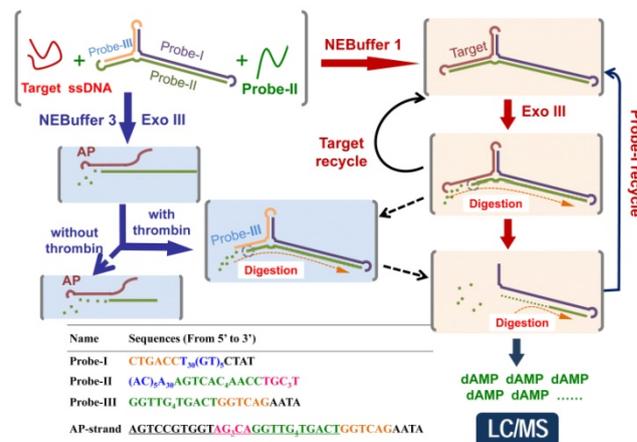
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**A label-free double amplification system has been developed by using a ternary DNA probe containing poly (adenine-thymine) sequence assisted by exonuclease III degradation. The method achieved more than 600-fold signal amplification and allowed sensitive detection of single-stranded DNA and thrombin at pM levels by using liquid chromatography/mass spectrometry.**

Well-designed DNA probes offer great opportunities for enhancing the detection sensitivity of single-stranded DNA (ssDNA) and other compounds that can specifically interact with ssDNA<sup>1-5</sup>. The DNA probe not only guarantees the specificity of the detection, but also enables signal generation or even amplification. A number of target amplification or target-recycling amplification systems have been reported in recent years<sup>6-21</sup>. The amplified products have been further measured by various methods such as fluorescent<sup>6-12</sup>, colorimetric<sup>13, 14</sup> and chemiluminescence<sup>15</sup> detection methods, surface plasmon resonance (SPR)<sup>16</sup>, and electrochemical detection methods<sup>17-21</sup>. However, little has been reported on signal amplification methods for mass spectrometry (MS) detection. Moreover, most of the available amplification systems only provide one signal per probe molecule, which limit the overall amplification fold<sup>6-9, 11-13, 15-18, 20, 21</sup>.

Herein, we demonstrate a double amplification concept by using a label-free ternary DNA probe which comprises a hybridized poly-adenine-thymine (poly-AT) segment with two free binding arms and an auxiliary strand. As depicted in Scheme 1, the near-3'-terminal sequence of Probe-I hybridizes with the near-5'-terminal sequence of Probe-II via 30 continuous adenine-thymine pairs and forms a conserved poly-AT hybrid with a protrudent 3'-end and two variable segments for recognition and binding to the target sequence. Probe-III was designed to be shorter than the target sequence near the 5'-terminal so that in the absence of the target ssDNA, all the 3'-ends of the ternary DNA probe are protrudent, which are inert to the addition of exonuclease III (ExoIII), a nuclease that can specifically identify the blunt or recessed 3' termini of double-stranded DNA (dsDNA) and stepwise remove mononucleotides from the 3'-hydroxyl end<sup>11, 21-23</sup>. Upon addition of the target DNA (see the red arrow with

NEBuffer 1 and right part of Scheme 1), it quickly displaces Probe-III and generates a recessed 3'-termini of Probe-II, thus immediately initiates the degradation of Probe-II by Exo III and releases a number of free 2'-deoxyadenosine-5'-monophosphates (dAMPs). Then, another Probe-II binds to the intact Probe-I and the target sequence, and the formed recessed 3'-termini is further digested by Exo III. After the recycling reaction, each target sequence leads to the degradation of multiple Probe-II, and produces hundreds of dAMP, which can be readily quantified with liquid chromatography/mass spectrometry (LC/MS).



Scheme 1. Schematic illustration of the working principle of the double amplification system. The inset table shows the sequences of Probe-I, Probe-II, Probe-III and the target sequence (AP-strand). The complementary parts are indicated in the same colour. The 29-nt sequence underlined in AP-strand is a thrombin binding aptamer<sup>4</sup>.

In the ternary DNA probe, the hybridized part between Probe-I and Probe-II serves as the reservoir of dAMPs, with the two free binding arms for recognition of the target sequence. Addition of Probe-III may promote the specificity of ssDNA detection via displacement hybridization. Based on Scheme 1, only Probe-II was

digested, while Probe-I and the target ssDNA were recycled. The ratio of Probe-I : Probe-II : Probe-III in the ternary probe solution was carefully optimized and the results were shown in Figure S1 in the Supporting Information (SI). Accordingly, the ternary probe solution were prepared by mixing Probe-I, Probe-II and Probe-III at a molar ratio of 1:10:1.

The poly (A) sequence in Probe-II plays key roles in the amplification system. Since Exo III prefers to digest dsDNA, it is very important to keep the poly (A) sequence in Probe-II stably hybridized with the poly-T strand in Probe-I until all the dAMP in the poly (A) was released. For this purpose, a ten-base-pair sequence ((AC)<sub>5</sub>-tail) was designed at the 5' termini of Probe-II. Figure S2 shows the amplification effects by using Probe-II with the (AC)<sub>5</sub>-tail. Attributed to the stabilization effect and additional dAMPs provided by the (AC)<sub>5</sub>-tail, both the signal intensity and the signal increase speed of dAMP enhanced dramatically. So Probe-II with the (AC)<sub>5</sub>-tail was employed to carry out the amplification and the reaction time was set at 15 min.

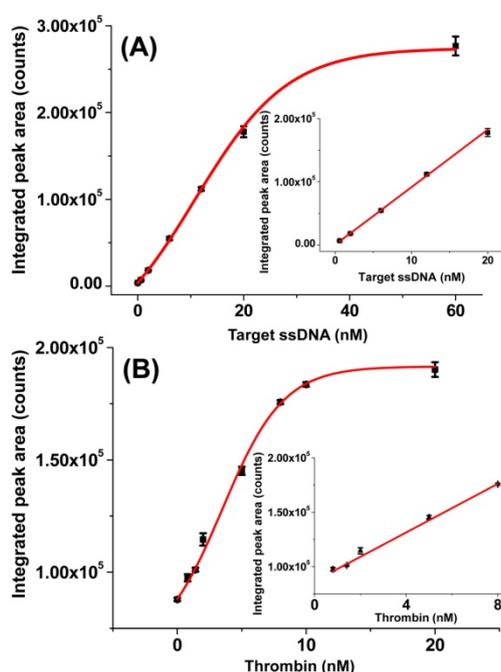


Figure 1. Detection of target ssDNA (AP-strand) (A) and thrombin (B) with the Exo III-assisted ternary DNA probe double amplification system. (A) Signal response curves of dAMP obtained at different concentrations of AP-strand ranging from 0 to 60 nM in NEBuffer 1 with the addition of 0.1 M NaCl. Inset shows the linear calibration curve ( $R^2=0.998$ ). (B) Signal response curves of dAMP to different concentrations of thrombin ranging from 0 to 20 nM in NEBuffer 3. Inset shows the linear calibration curve ( $R^2=0.997$ ). The concentrations Probe-I, Probe-II, Probe-III and Exo III are 20 nM, 200 nM, 20 nM and 0.2 U· $\mu$ L<sup>-1</sup>, respectively. The concentration of AP-strand used for thrombin detection is 20 nM.

Figure 1A shows the signals of dAMP obtained at different concentrations of the target ssDNA (AP strand) using the ternary-probe amplification system. The corresponding chromatograms and mass spectra were all shown in Figure S3. The average integrated peak area of dAMP detected by LC/MS showed a good linear

relationship with the concentration of AP-strand in the range from 0.6 nM to 20 nM, with a limit of detection (LOD) of 370 pM (S/N = 3). In comparison with the detection limit of dAMP by LC/MS, 225 nM<sup>24</sup>, a 608-fold enhancement of sensitivity was achieved by the amplification system for DNA sequence detection.

In the conventional Exo III-based amplification methods using fluorescent probes, the signal offered by each fluorescent probe depended on the fluorescence difference between the off and on states of the fluorophore, which are usually from 10 to 100 folds<sup>9-11</sup>. By contrast, the signal offered by each ternary DNA probe was determined by the number of released dAMPs from the poly (A) sequence, which was estimated to be more than 30 for each Probe-II molecule. This may be further enhanced by increasing the number of adenines in Probe-II. Thus, in combination with the Exo III-assisted target-recycling process, the ternary probe may offer much higher amplification fold than the fluorescent probes.

Next, we attempted to extend the amplification system to detect protein analytes. The near-5'-terminal 29-nt sequence in AP-strand is actually an aptamer against thrombin, an important serine protease in the blood coagulation cascade<sup>4</sup>. So we further tested whether the amplification system can be employed for the detection of thrombin. To this end, we incubated different amounts of thrombin with the AP-strand solution in NEBuffer 3, in which the aptamer sequence could interact with thrombin by forming a G-quadruplex structure<sup>4</sup>. The AP-strand/thrombin solution was mixed with the ternary probe solution, to which Exo III were immediately added. Interestingly, instead of inhibiting the amplification reaction of the AP-strand, addition of thrombin significantly enhanced the dAMP signals in comparison with the no thrombin control solution. As shown in Figure 1B and Figure S4, the dAMP signals increase with the increase of thrombin concentration. The linear working range was from 0.8 nM to 8 nM, and the LOD of thrombin was 280 pM (S/N = 3), which is comparable with the electrochemical detection methods and much lower than the fluorescent and colorimetric methods<sup>14, 21, 25</sup>.

To find out the possible mechanisms for above results, we performed native polyacrylamide gel electrophoresis (PAGE) analysis of reaction solutions with different compositions. The results are shown in Figure 2A and Figure S5. A detailed description of the PAGE results is presented in the SI. In NEBuffer 1, most of the target ssDNA (AP strands) formed the desired AP/Probe-I/Probe-II complex in the mixture solution, thus the amplification reaction was effectively initiated and lead to rapid increase of the dAMP signals. It is worth mentioning that Probe-III plays a notable role in accelerating the amplification process. Figure S6 provided further experimental results on this point.

In NEBuffer 3, however, the formation of AP/Probe-I/Probe-II complex is significantly affected by the secondary structure of AP strands and their interactions with thrombin. With the addition of Exo III, Probe-II in the AP/Probe-II hybrid is first digested to short fragments. The resultant Probe-II fragments 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 are released. In the presence of thrombin, with the digestion of Probe-II in the AP/Probe-II hybrid, the shortened Probe-II fragment may 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 (see the blue arrow with NEBuffer 3 and left part of Scheme 1).

The specificity of the method towards thrombin over other proteins such as myoglobin, lysozyme, cytochrome *c* and insulin were investigated and the results are shown in Figure 2B. As can be seen, the signal response of dAMP to thrombin is remarkably higher than to other tested proteins, indicating that the increase of dAMP signals was specifically induced by the interactions between thrombin and the AP-strand.

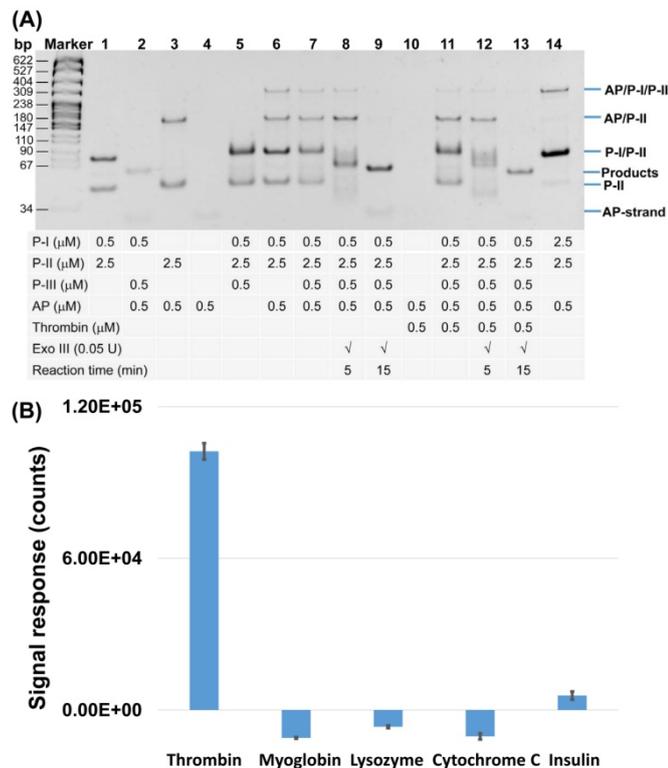


Figure 2. (A) Native PAGE analysis of the amplification reaction solutions in NEBuffer 3 for the detection of thrombin. (B) Selectivity of the amplification system to thrombin over other proteins. The signal response of each protein represents the difference between the integrated peak areas of dAMP obtained in the presence and absence of the protein (20 nM).

Direct quantification of large biomolecules such as DNA or proteins by MS usually suffers from complicated sample pretreatment, interference of background, expensive internal standard isotopic substance and heavy data interpretation<sup>26, 27</sup>. By incorporating the above specific amplification system, the DNA or protein analytes can be indirectly quantified by measurement of the released small molecule dAMP at a much higher concentration. Moreover, the presented method has great potential to be combined with ambient MS, such as paper spray MS, which may offer a rapid, label-free, and sensitive way to analyse complex biological samples<sup>28</sup>.

In conclusion, we have developed a label-free ternary DNA probe-based double amplification method for sensitive detection of ssDNA and thrombin. The method achieved more than 600-fold amplification and enables quantification of ssDNA and thrombin at pM concentration level by using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). The ternary DNA probe

can be flexibly adjusted to detect different target DNA sequences by simply changing the sequences of the two free binding arms of Probe-I/Probe-II hybrid. Furthermore, the method has great potential for further application to detect a wide variety of analytes via their interactions with DNA sequences.

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## Notes and references

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