Electronic Supplementary Information

The Real-time PCR for Sensitive Protein Detection by Target-induced Intermolecular Hybridization

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Reagents and Material. All oligonucleotides used in this work were supplied and HPLC purified by SBS Genetech. Co. Ltd. as seen in Table S1. The italic portions in A probe and B probe were aptamer sequences of thrombin. The boldface in A probe and B probe were the complementary nucleotides. According to the reference[1], the complementary regions of probes with 10-, 8-, or 6-bp were optimized and 8-base duplex was found to be optimum. So, the experiments in our work were carried out with an 8-bp complementary region. The different groups of probes around 75-, 80-, or 85-nucleotides also have been optimized (data not shown), but negligible effects on the result were obtained. We selected two affinity probes around 80 nucleotides each because it was convenient for gel electrophoresis to analyze PCR products（≈ 100 bp).

Table S1. Sequences of single- strand probes and primers

Name	Sequence (5′to 3′)
A probe	AATACCCGATTGCAGTACGACTCTC **CACAAGCC** TTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTT *GGTTGGTGTGGTTGG* (80 nt)
B probe	CG *AGTCCGTGGTAGGGCAGGTTGGGGTGACT* CGATCAATGTGACT ACTCGTGGCTAGTGTTTTTTTTTTTTTT **GGCTTGTG** (82 nt)
Primer 1 (P1)	AATACCCGATTGCAGTACGACTC (23 nt)
Primer 2 (P2)	GAGTCCGTGGTAGGGCAGGT (20 nt)

Klenow Fragment (exo$^-$) polymerase and dNTPs were purchased from MBI Fermentas Crop. Human thrombin (10 U/mg) was obtained from Ding Guo Co. Ltd. (Beijing, China). Bovine serum albumin (BSA) was purchased from Westang (Shanghai, China). Trypsin and bovine thrombin were purchased from Sigma-Aldrich Inc. Human serum was offered by Qingdao municipal Hospital in China. Power SYBR Green PCR Master Mix kit was purchased from Applied Biosystems. All solutions were prepared with doubly distilled water.

Intramolecular Hybridization-dependent Polymerase Reaction Assay. The affinity

probes were denatured at 90℃ for 5 min and rapidly cooled to 0℃. 2-μL aliquots containing different concentrations of thrombin were mixed with 2 μL 4.0×10^{-10} M each affinity probe in the reaction buffer containing 20 mM Tris-Ac (pH 7.9), 50 mM KAc, 10 mM Mg $(Ac)_2$ and 1 mM DTT at 37℃ for 20 min. Then, 0.5 U polymerase and 0.9 μL 2.5 mM dNTPs were added to the resulting mixture in a total volume of 10 μL at 37℃ for 20 min and subsequently heated to 90℃ to inactivate the Klenow Fragment (exo^-) polymerase.

Detection of Thrombin by Real-time PCR. A 2.5-μL aliquot of the polymerase reaction mixture was used as template for real-time fluorescence quantitative PCR (qPCR). The PCR reaction mixture included 2.5 μL of template, 25 μL 2×Master mix, and 1.0 μL10 μM of each primer in final volume 50 μL. The PCR reaction mixtures were transferred to an ABI StepOne real-time PCR Instrument (Applied Biosystems, USA) for the cycling program consisting of an initial denaturation at 95℃ 5 min followed by 40 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 10 s. All the reactions were run in triplicates, and the same reaction mixtures without thrombin were used as negative controls. The cycle thresholds (C_t) for deriving sensing qPCR were generated from PCR containing thrombin from 1.0×10^{-7} M to 1.0×10^{-12} M.

Selectivity of the assay. To prove the selectivity of our method, the samples that contained the final concentration of 1.0×10^{-9} M bovine serum albumin (BSA), trypsin or bovine thrombin were tested as the selectivity experiments, respectively. The assay procedures for BSA, trypsin and bovine thrombin were the same as that for human thrombin, except for using BSA, trypsin and bovine thrombin instead of human thrombin.

Application of the Approach in Human Serum. Thrombin was further tested in the human serum without any pre-treatment to illustrate the feasibility of the approach. Serum, diluted 2

times, was tested alone or spiked with thrombin concentration range from 1.0×10^{-8} M to 1.0×10^{-11} M.

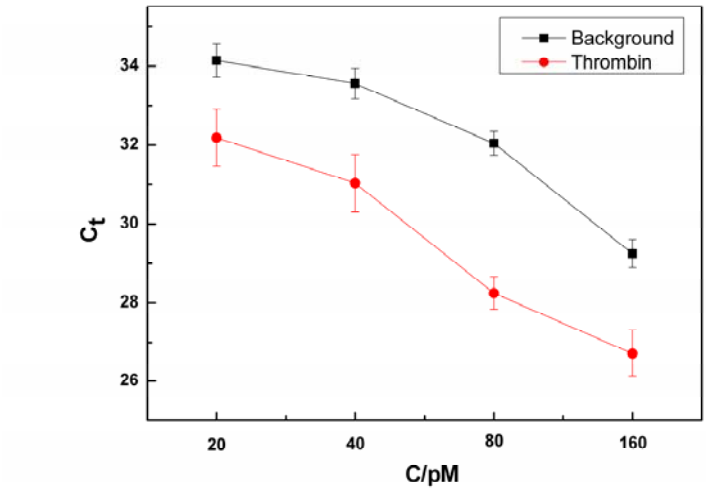


Figure S1. Optimization of probe concentration for the assay. The ■ showed the target-independent background while ● represented C_t values in the presence of 1.0×10^{-10} M thrombin.

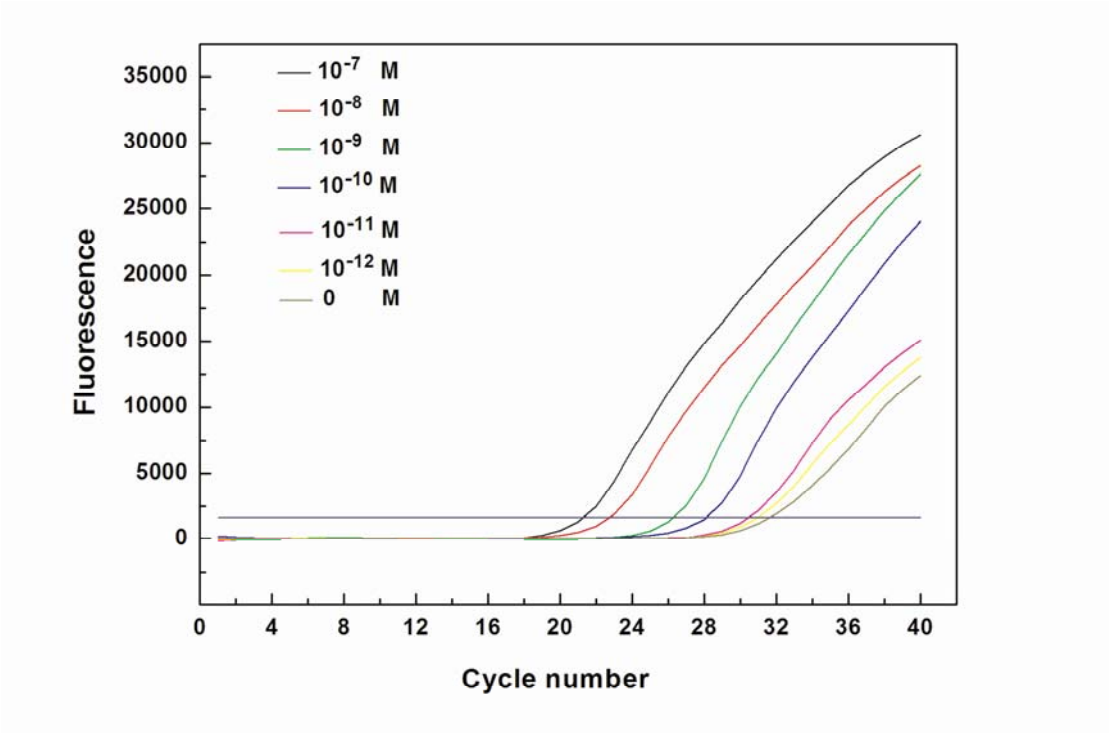


Figure S2 Amplification curves observed by real-time PCR using SYBR Green Ⅰ. 10-fold serial dilutions of thrombin (the concentration range from 10^{-7} to 10^{-12} M) were used and the negative control was without thrombin. The concentrations of two affinity probes were 8.0×10^{-11} M, respectively.

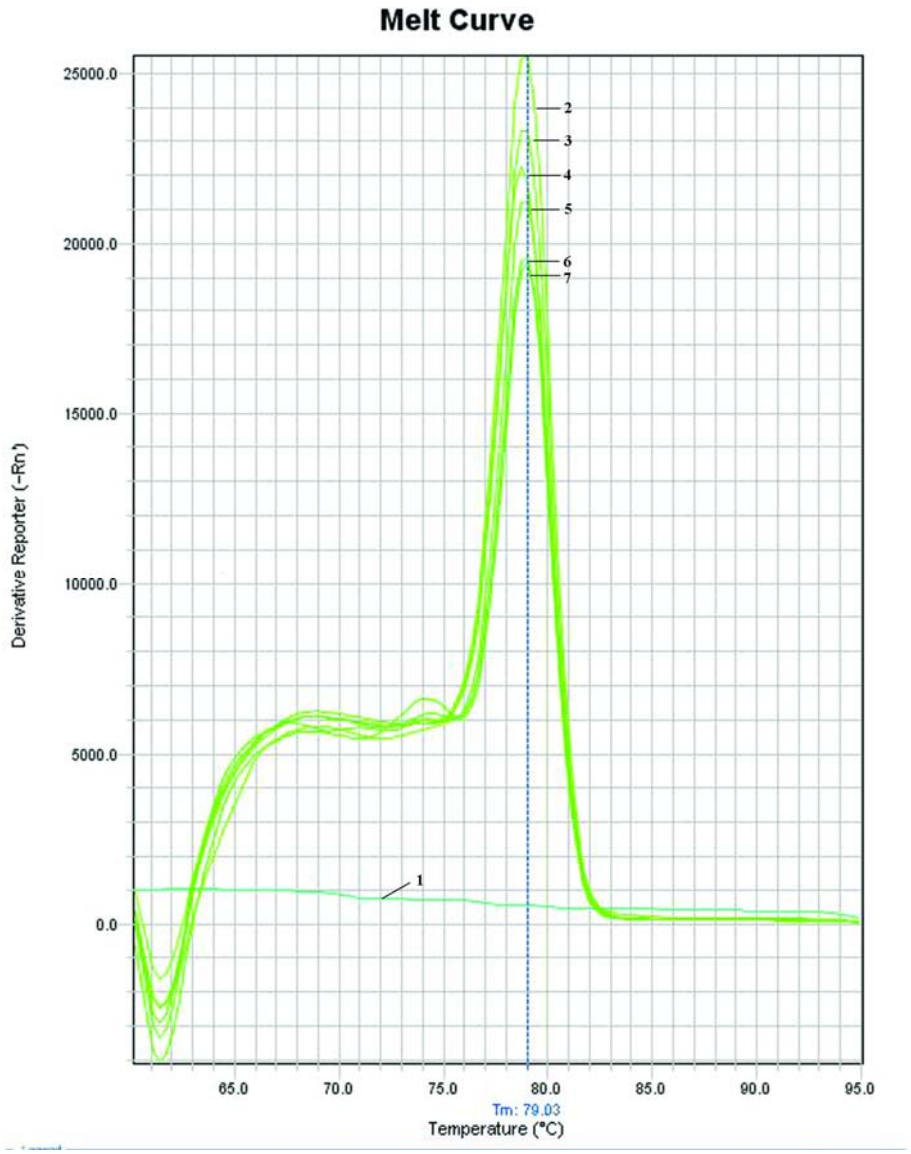
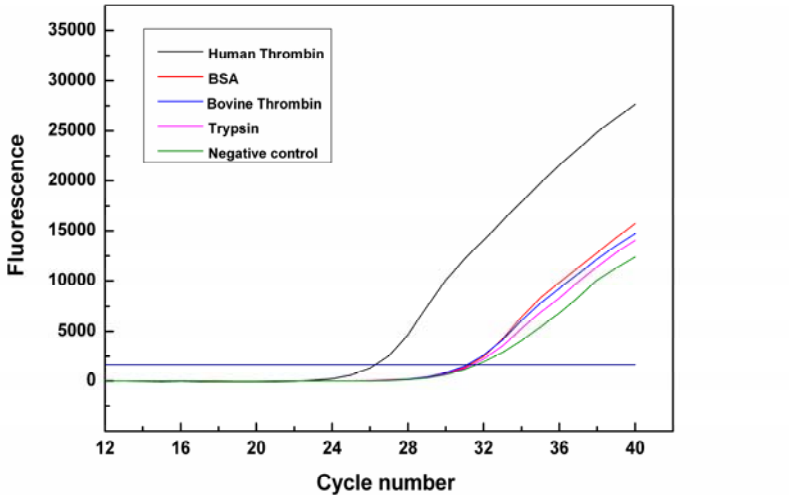


Figure S3 The melting curves of real-time PCR using SYBR Green Ⅰ. 1. Negative control; 2-7. Thrombin; The melting curves of negative control and different concentrations thrombin showed that no primer-dimer or other byproduct amplification happened.

A.



B.

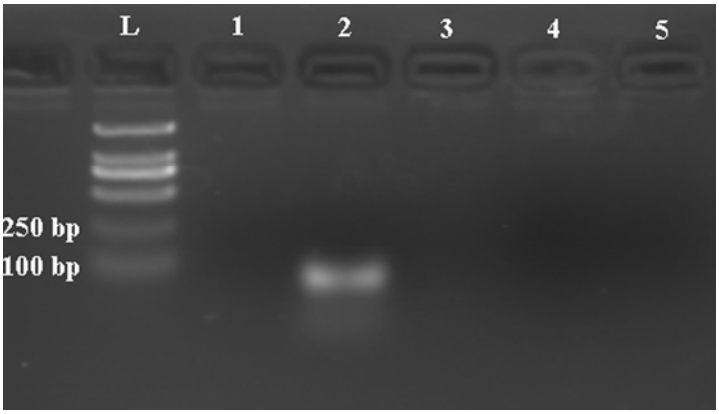


Figure S4. Specificity analysis of the assay. A. C_t values difference between different proteins. The result showed the C_t values for BSA, trypsin and bovine thrombin were close to the C_t value for the negative control. B. Specificity analysis using 2.5% agarose gel electrophoresis of qPCR amplification products for different proteins. Lanes: L. 2000 bp DNA ladder; 1. negative control; 2. 10^{-9} M human thrombin 3. 10^{-9} M BSA; 4. 10^{-9} M bovine thrombin; 5. 10^{-9} M trypsin There were no bands with BSA, bovine thrombin or trypsin, while there was the correctly size band in the thrombin sample, which showed no significant effect on thrombin detection for these proteins.

Reference

1. D.J. Gorin, A.S. Kamlet and D.R. Liu, *J. Am. Chem. Soc*., 2009, **131**(26), 9189-9191.