

Fluorescence Detection for DNA Using Hybridization Chain Reaction with Enzyme-amplification

Shuyan Niu, Yu Jiang, Shusheng Zhang*

*Key Laboratory of Eco-chemical Engineering, Ministry of Education; College of
Chemistry and Molecular Engineering, Qingdao University of Science and
Technology, Qingdao 266042, P. R. China*

Chemicals

Streptavidin-coated MBs (mean diameter = 3~4 μm ; 10 $\text{mg}\cdot\text{mL}^{-1}$) magnetic beads (diameter) were ordered from Tianjin BaseLine Chromtech Research Centre and reaction buffer consisted of 50 mM sodium phosphate, 0.5 M NaCl, pH 6.8. 1 $\times$ TAE buffer consisted of 40 mM Tris-acetate and 1 mM EDTA, pH 8.0.

The 4-hydroxyphenylaceticacid (p-HPA) was obtained from Johnson Matthey Company (Shanghai, China). 0.2 M Britton – Robinson (B – R) were used as incubating and washing buffer for the enzyme reaction (pH 8.0). The substrate solution containing p-HPA was prepared by dissolving 1.3 mg p-HPA in 1 mL 0.2 M B – R (pH 8.0) buffer solution containing 0.6 mM H_2O_2 , 1.3 M isopropanol.

All synthetic oligonucleotides were purchased from SBS Genetech Company (Beijing China):

Target (T1): 5'-CCCTGGGCTCAACCTAGGAATCGC-3'

Single mismatch target (T2): 5'-CCCTGGGCTCAACCTAGCAATCGC-3'

Three bases mismatch target (T3): 5'-CCCTGGGCTCAACCTTCCAATCGC-3'

* Corresponding author. Tel.: +86-532-84022750; Fax: +86-532-84022750.

E-mail: shushzhang@126.com

Bio-H1: 5'-**biotin** GCG ATT CCT AGG TTG AGC CCA GGG TTT TTT CAC
AGT CCC TGG GCT CAA CCT AGG-3'

H1: 5'-**FAM**-GCG ATT CCT AGG TTG AGC CCA GGG TTT TTT CAC AGT
CCC TGG GCT CAA CCT AGG-3'

Bio-H2: 5'-CCC TGG GCT CAA CCT AGG AAT CGC TTT TTT CCT AGG TTG
AGC CCA GGG ACT GTG-**biotin**-3'

H2: 5'-CCC TGG GCT CAA CCT AGG AAT CGC TTT TTT CCT AGG TTG AGC
CCA GGG ACT GTG-**Fluorescein**-3'

(*In the hairpin sequences, loops are underlined and sticky ends are overlined and the italicized bases indicate those mismatched with target; As to the sequences of Bio-H1, the sticky ends represent fragment 'a' of Scheme 1 and Scheme 2, and the last 6 bases of the loop represent fragment 'c'; while in the sequence of Bio-H2, the sticky ends represent fragment 'c*', and the first 6 bases of the loop represent fragment 'a*')

All oligonucleotides were dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer) and kept frozen. Other chemicals employed were all of analytical reagent grade and deionized water was used throughout the experimental work.

Apparatus

A Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) equipped with 1 cm quartz cells was used for fluorescence measurements. The pH of all solutions was measured by a Model pHs-3D digital acidometer (Rex Electric Chemical Products Department, Shanghai Precision Scientific Instrument Co., Ltd.). Gel

electrophoresis was carried out on the EPS300 electrophoretic apparatus (Shanghai Tanon Science & Technology Co., Ltd).

Gel electrophoresis

Stock solutions of T1, Bio-H2, and Bio-H1 were diluted in reaction buffer to three times their final concentrations (see Fig.1), and 10 μ L of each species was mixed, in the order of T1, Bio-H1, and Bio-H2 to a total reaction volume of 30 μ L. Reactions were incubated at room temperature for 24 h before running 1 μ L of each product on a gel. The 1% agarose gels contained 0.5 μ g of ethidium bromide per ml of gel volume and were prepared by using $1 \times$ TAE buffer. Agarose gels were run at 100 V for 30 min and visualized under UV light.

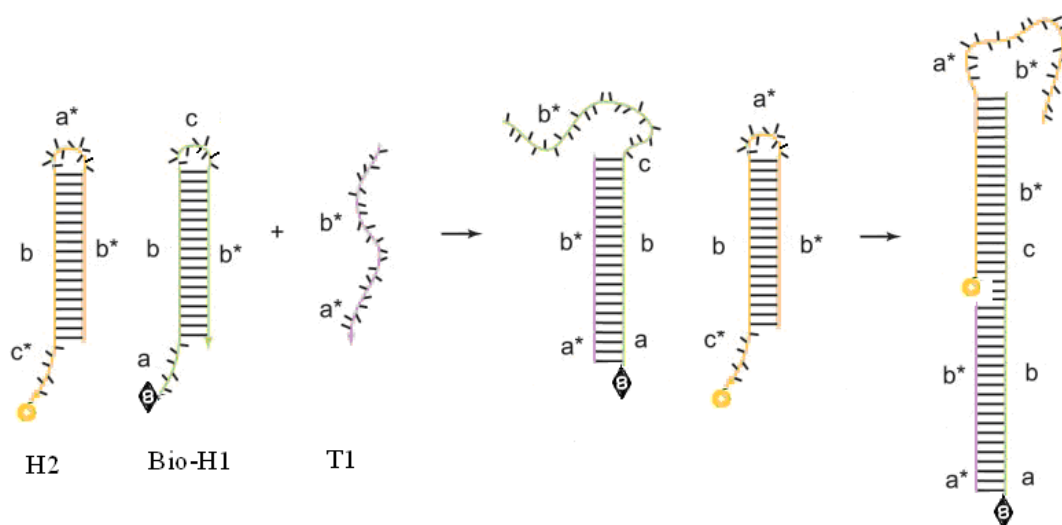
Preparation of the streptavidin-coated MBs and hairpin oligonucleotides

Streptavidin-coated MBs were prepared according to the literature(1). Briefly, 5 μ L of streptavidin-coated MBs was washed twice with 100 μ L washing buffer of Tris buffer (pH 8.0) with 100 mM Tris, 0.1% (v/v) Tween 20, and 1 M NaCl before use. All the hairpin oligonucleotides were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use.

Fluorescence detection with fuels labeled with FAM

Stock solutions of T1, Bio-H1, H1 and H2 were diluted in reaction buffer to the final volume of 100 μ L with their final concentrations as follows: 2.0×10^{-8} M Bio-H1; 1.0×10^{-7} M H1; 1.0×10^{-7} M H2. Reactions were incubated at room temperature for two hour before reacting with the prepared MBs. After the suspension was incubated for 1 h at 20°C with mechanical shaking, the solution was separated by

magnetic separator and then diluted by reaction buffer to a volume of 600 μL . The fluorescence measurements were carried out on a Hitachi F-4500 fluorescence spectrophotometer in the wavelength range from 400 nm to 700 nm at a scan speed of 1200 $\text{nm}\cdot\text{min}^{-1}$ with excitation wavelength at 460 nm.



Scheme S1 Schematic representation of the HCR process

The proportion of Bio-H1 and H1

Since the Bio-H1 and the H1 has the same sequence, they played an equal role as fuels in the DNA nanomachine. However, too little Bio-H1 will lead to a negative effects on the reactions between HCR products and MBs, while too much Bio-H1 will make the products a low fluorescence response. We studied the proportion of the two to achieve the best fluorescence intensity efficiency of the system. Figure S1 show that when the proportion was 1:5, the system had the largest decrease after the

magnetic separation. (ΔF was the reduction of fluorescence intensity of the system)

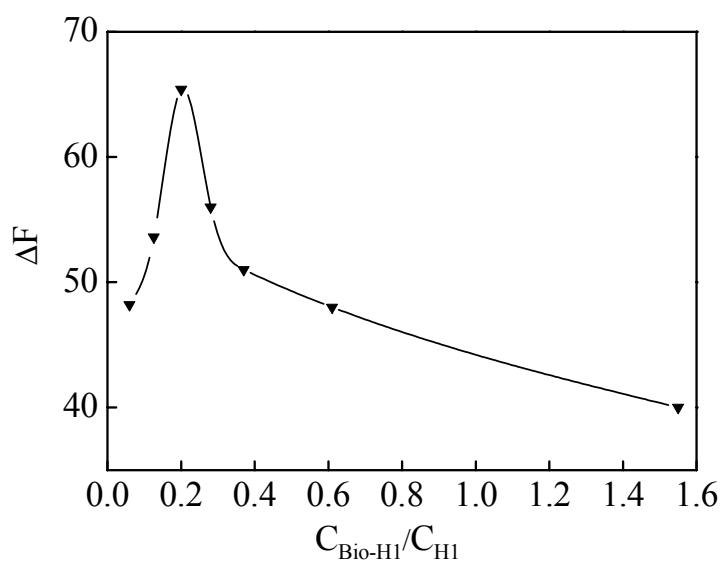


Figure S1 Effects of the proportion of Bio-H1 and H1 on the fluorescence intensity reduction where the H2 was 0.1 μM and the total concentration of Bio-H1 and H1 maintained to be 0.1 μM

The proportion of H1 and H2

In order to obtain the largest growth efficiency, we explored the proportion of the two kinds of fuels. Figure S2 shows that when the proportion arrives at 1.2:1, the reduction of the fluorescence intensity obtains the maximum after reacting with target of the same concentration. (ΔF was the reduction of fluorescence intensity of the system)

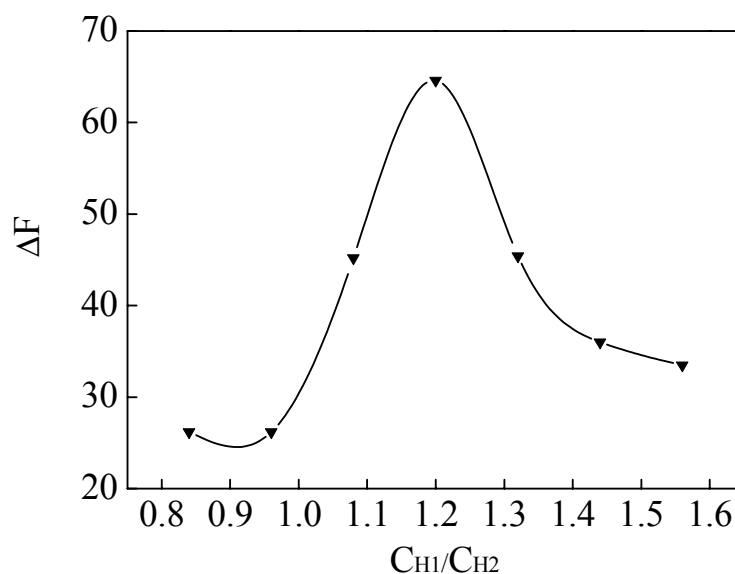


Figure S2 Effects of the proportion of the two kinds of fuels on the fluorescence intensity reduction where the proportion of Bio-H1 and H1 was 1:5 with their concentration maintaining to be 1.2 μ M

HCR system for fluorescence detection

Since the structure of the fuels obstructed the hybridization, the different labeled hairpins won't combine together without initiator, which therefore will not lead to the reduction of the fluorescence intensity in the system after magnetic separation. Initiators determined the number of the active sequence, and consequently determined the length of molecular and combination efficiency of the two fuels. Thus as the initiators increased in the system, the reduction of the fluorescence intensity become larger after the magnetic separator as shown in Figure S3.

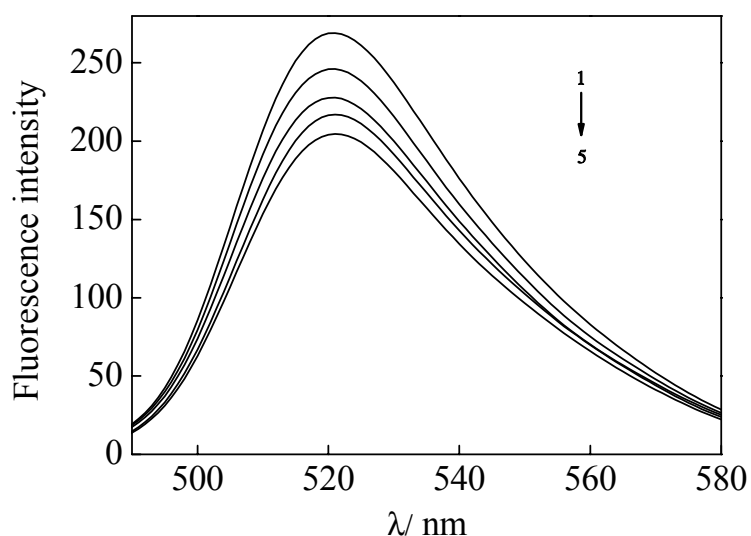


Figure S3 The reduction of the fluorescence response with different concentrations of target DNA in the HCR system. (1) 0.00 M; (2) 1.00×10^{-11} M; (3) 5.00×10^{-11} M; (4) 7.00×10^{-11} M; (5) 9.00×10^{-11} M

Procedure of fluorescence quantification with enzyme-amplification

100 μ L 1.0×10^{-7} M Bio-H1 was added into a 1.5 mL sample tube with 5 μ L prepared MBs. After mechanical shaking for 30 min at 37°C to make sure that the surface of the MBs has been covered fully with Bio-H1, the solution was removed and 100 μ L reaction solutions with different concentrations of T1 was added. After mechanical shaking for 30 min at 30°C, we removed the suspension and added 100 μ L reaction solution with 1.0×10^{-7} M Bio-H1 and 1.0×10^{-7} M Bio-H2. After incubation at 30°C for 2 h, suspension was removed (every magnetic separation above includes a washing step with washing buffer). Then 300 μ L ultrapure water containing 20 μ g mL⁻¹ avidin-HRP for 35 min at 37 °C. After being rinsed with PBS (20 mM

sodium phosphate, pH7.4) to remove unbound avidin-HRP, 1.00 mL substrate solution containing p-HPA was added and incubation lasted for 1.5 h. After the magnetic separated, the reacted solution was transferred into 1.0 cm quartz cells and the fluorescence measurements were carried out on a Hitachi F-4500 fluorescence spectrophotometer in the wavelength range from 340 nm to 520 nm at a scan speed of $1200 \text{ nm}\cdot\text{min}^{-1}$ with excitation wavelength at 320 nm. Background fluorescence had been subtracted for each value.

Although the HCR system with different modifying fuels could demonstrate the growth of the double strands quickly, the high background will affects the results. Compared to utilization of the fluorescence signal group modifying fuels to achieve the quantitative analysis, the application of the biotin labeled fuels and enzyme-amplification will increase the sensitivity by about 100-fold. Meanwhile, the avidin-HRP and fuels hardly have physisorption with MBs, thus the blank value of the system is neglectable.

The effect of reaction time for the HCR system with enzyme-amplification

With the target concentration $1.0 \times 10^{-14} \text{ M}$, the incubation time was changed to 0 min, 10 min, 30 min, 60 min, 120 min, 240 min, respectively. The fluorescence reponses were demonstrated in the Figure S4.

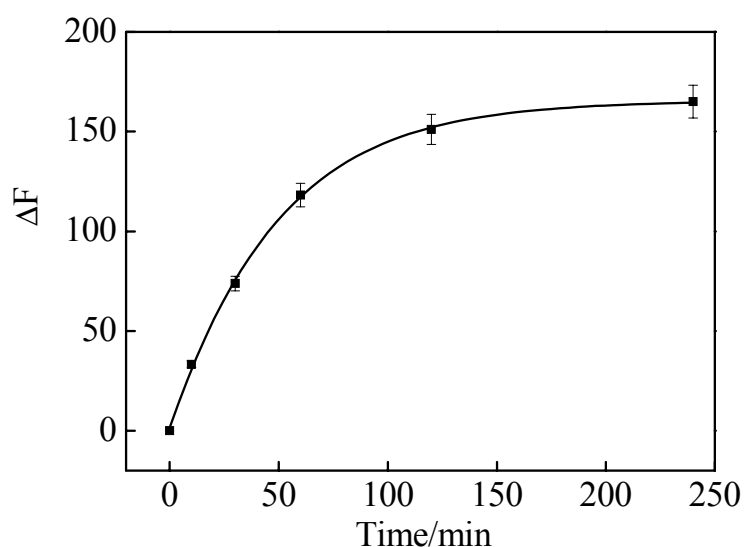


Figure S4. The fluorescence responses of the HCR system with enzyme-amplification with different reaction time of HCR operation. The concentration of the target is 1.0×10^{-14} M

The Magnification of the HCR

In order to investigate the length of the product strand after HCR and magnification of the HCR system, we study the fluorescence response of the HCR system with only one kind of fuels, namely Bio-H2, with enzyme-amplification. We obtained a relation curve as is shown in the Figure S5. The regression equation was $\Delta F = 19.92 + 2.76(C_{T1}/pM)$ (ΔF was the intensity of bi-p,p'-4-hydroxyphenylacetic acid, background fluorescence was subtracted; C_{T1} was the concentration of the target) and calculate the detection limit as 2.65×10^{-14} M. Therefore, we draw the conclusion that the HCR has got about 30-fold magnification during the analysis.

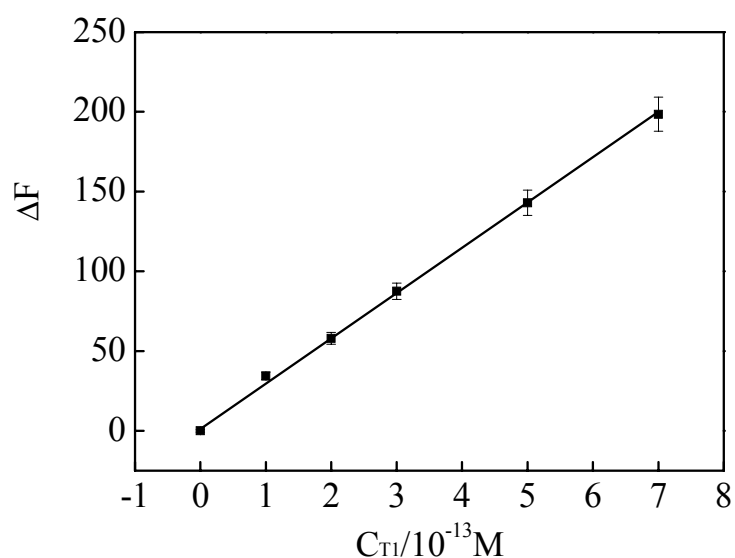


Figure S5 The relation curve of fluorescence intensity and different amounts complementary target DNA with only Bio-H2 in the HCR system.

The Comparison with other DNA detection.

As we can see from the table S1, one-step amplification² with HRP is not sensitive enough for the biosensors. Most amplifications are involved with nanoparticles, need complex modification operation and must take care of the reaction conditions to prevent the aggregation.

Table S1. Comparison between the Proposed Assay and Other Reported Techniques for the Determination of DNA Hybridization

Label	Detection techniques	Detection limit of ssDNA	Ref.
Avidin-HRP	Fluorescent	1 pM	2
Nanomagnetic beads	Chemiluminescent	1 fM	3
AuNPs	chronocoulometric	10 fM	4
liposome	liposome-amplified electrochemical	50 fM	5
HCR with enzyme-amplification	Flourescent	0.8 fM	This method

Reference

- (1). Selvaraju, T., Das, J., Jo, K., Kwon, K., Huh C.H., Kim, T. K., and Yang, H., *Langmuir*, 2008, 24, 9883
- (2) Niu, S.Y., Li, Q.Y., Ren, R., Zhang, S.S. *Biosens. Bioelectron*, 2009, 24, 2943
- (3) Wujian Miao, and Allen J. Bard. *Anal. Chem.*, 2004, 76, 5379
- (4) J. Zhang, S. P. Song, L. Y. Zhang, L. H. Wang, H. P. Wu, D. Pan and C. H. Fan, *J. Am. Chem. Soc.*, 2006, 128, 8575.
- (5) F. Patolsky, A. Lichtenstein and I. Willner, *Angew. Chem. Int. Ed.*, 2000, 39, 940.