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Colorimetric detection of microRNA based hybridization chain reaction for signal amplification and enzyme for visualization

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ABSTRACT

MicroRNAs (miRNAs) have key roles in gene expression and can be employed as biomarkers for early diagnosis of various diseases, especially cancers. Detection of miRNAs remains challenging and often requires detection platforms. Here, a horseradish peroxidase (HRP)-assisted hybridization chain reaction (HCR) for colorimetric detection of miR-155 was described. In the presence of target miRNA, the capture probe immobilized on the microplate sandwiched the target miR-155 with the 3′ end of the reporter probe. Another exposed part of the RP at the 5'end triggered HCR producing double-stranded DNA polymers with multiple fluorescein isothiocyanates (FITC) for signal amplification. Finally, multiple HRP molecules were immobilized onto the long-range DNA nanostructures through FITC/anti-FITC monoclonal antibody interactions on the microplate for visualization by tetramethylbenzidine/H_2O_2 system and the colorless substrate turned into the blue product. To obtain accurate data, the absorbance at 450 nm was calculated by microplate reader. The detection limit was 31.8 fM (3.18 amol). Furthermore, this biosensor showed high specificity and was able to discriminate sharply between target miRNA and mismatched sequences. And this approach could be easily applied to the detection of miR-155 in serum sample, thereby ascribing it for a wide application.

Introduction

MicroRNA is a single chain, evolutionarily highly conserved, endogenous, non-protein-coding small RNA molecule (19–23 nucleotide), which regulates the gene expression [1]. Presently, more than 2000 miRNA targets have been discovered that regulate more than 30% of the human genome [2,3]. Many studies found that human disease occurrence, early development, and immune system dysfunction are associated with miRNA expression [4,5]. MiRNAs have become a new generation of diagnostic markers. Overexpression of miRNA-155 (miR-155) is observed in multiple types of cancers, such as chronic lymphocytic leukemia and hematopoietic malignancies (malignancies of the hematopoietic system), colon, cervical, lung, breast, pancreas, thyroid cancer, and other solid tumors [6,7]. Therefore, miR-155 can be used as a biomarker to predict tumor and disease progression.

Owing to their small size, low abundance, and high homology between the phylogeny of human miRNAs, detection of miRNAs is challenging [8]. Northern blotting, microarray, and quantitative real-time PCR (RT-qPCR) are the conventional strategies providing ideal platforms for miRNA expression profiling. However, northern blotting has a low sensitivity and specificity for the detection of miRNA. It is laborious and time-consuming, and radioactive isotope labeling is not environmentally friendly. RT-qPCR and microarray have adequate detection limits but require expensive equipment and materials, which limits their use [9]. In recent years, fluorescence [10] and surface plasmon resonance (SPR) [11] have been used for miRNA detection because of their precise quantitative assessment. But they also require specialized laboratory conditions, expensive equipment.

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Enzyme-linked assays have been routinely used for miRNA detection as they can cost-effectively amplify the detection signal with relative ease as a consequence of the rapid conversion of substrates into products that can be readily visualized by the naked eye without requiring additional instruments [7]. However, they often suffer from low sensitivity, especially during the assessment of real samples. To enhance the detection sensitivity, several groups have employed nucleases to amplify the microRNAs detection signals [12]. Moreover, the nuclease-assisted target recycling renders the experimental process complicated and the detection process cost-ineffective.

HCR is a new type of non-enzymatic nucleic acid isothermal amplification technology. At room temperature (RT) and in the presence of the initiator sequence, two stable hairpin probes self-assemble to extend the double-stranded DNA polymers, which avoids using enzymes under stringent conditions [13–15]. Extended double-stranded polymers, and the signal molecules of fluorescence [16–18] electrochemical techniques [19–21] etc can be detected.

Here, we present a sensitive and visual detection platform for miRNA detection based on HCR in conjunction with horseradish peroxidase (HCR-HRP). MiR-155 is a model target in this study. HCR can produce modified long dsDNAs with multiple FITCs, through which anti-FITC monoclonal antibody-HRP (anti-FITC-HRP) conjugation can be attached for the visual and measurable signal. This method exhibited high sensitivity to target miRNA with the limit of detection (LOD) of 31.8 fM. Furthermore, the assay was also capable of discriminating the target miRNA from even a single base mismatched nucleic acid, which is promising in medical diagnosis and biological therapy in the future.

Materials and methods

Reagents and chemicals

All DNA sequences (Table 1) were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). All oligonucleotides were dissolved in sodium phosphate sodium chloride buffer solution (SPSC; 150 mM Na_2HPO_4, 0.75 M NaCl, pH 7.4) to a storage concentration of 10 μM. Streptavidin (SA)-coated 96-well plates (catalog no.15120), 4,4′-diamino-3,3′,5,5′-tetramethylbenzidine (TMB) substrate (product code: N301), ethidium bromide, bovine serum albumin, acrylamide, N,N-methylene bisacrylamide, ammonium persulfate, and tetramethylethylenediamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (HyClone) were purchased from Thermo Fisher Scientific (Waltham, MA). Anti- FITC-HRP mouse monoclonal antibody (catalog no. MAB045P) was purchased from Millipore (Billerica, MA, USA). All the other reagents used in the experiments were analytical grade. Nuclease-free water was used for all aqueous solution preparations. The sequences adopted from the literature are listed in Table 1.

Polyacrylamide gel electrophoresis

H1-FITC (1 μM) and H2-FITC (1 μM) were heated to 95 °C for 5min separately and then cooled to RT for 1 h before use. Ten microliters of different concentrations of RP were incubated with the same volume of H1-FITC and H2-FITC at RT for 2 h. A gel stock solution (30%, m/v) was prepared by dissolved 29 g acrylamide and 1 g N, N-methylene bisacrylamide in 100 mL purified water. The separating gel was prepared by mixing 3 mL gel stock solution, 6.890 mL of 5 × TBE buffer (0.45 M Tris, 0.45 M Boric acid, 10 mM Na_2EDTA, pH = 8.3), 100 μL ammonium persulfate and 10 μL of tetramethylethylenediamine. The native gel was run at 140 V for 1 h with 10 μL sample/well, stained for 15 min with 0.5 μg/mL ethidium bromide, and visualized under UV light.

Best ratio of hairpin-FITC/hairpin probes

Four groups of probes (H1-FITC, H2-FITC, H1, and H2) were diluted to 1 μM, respectively, heated at 95 °C, and cooled to RT to form the hairpin structure. Ten microliters of the reaction systems of FITC-labeled and unlabeled hairpin probes with different volume ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) were separately established. 10 μL of the RP (0.2 μM) was added to a final volume of 30 μL. The reaction mixtures were incubated at room temperature for 2 h. The bands of dsDNA polymers formed via HCR were analyzed on native polyacrylamide gel (9%, m/v) electrophoresis.

MiR-155 detection by HCR-HRP on the plate

SA-coated 96-well plate was used. Each well of the plate was incubated with 100 μL biotin-labeled capture probe (CP, 0.5 μM) in SPSC buffer at RT for 1 h. Subsequently, the microwells were washed thrice with washing buffer (25 mM Tris, 150 mM NaCl, 0.1% (m/v) bovine serum albumin, 0.05% (v/v) Tween 20) to remove the unreacted CP. Then, 100 μL miR-155 of different concentrations from 100 fM to 100 nM were added to the microplate and incubated at RT for 30 min. Thus, the target miR-155 was captured by hybridization with CP to form a duplex. After washing away the unreacted target miR-155, 100 μL HCR products from the mixture of RP (0.2 μM), H1 and H2 (The volume ratio of Hairpin-FITC/Hairpin was 9:1 and the concentration of each hairpin probe was 1 μM separately) were added to each well and maintained at RT for 30 min to form a sandwich of "CP/miRNA/RP-HCR". Next, 100 μL of HRP-Ab solution (1:2000 in SPSC buffer) was added to each well, and the plate was agitated on a shaker for 30 min. The plate was washed thrice to remove the unreacted HRP-Ab, following which, 100 μL of TMB substrate solution was added to each well and incubated in the dark at RT for 10 min. The colorless substrate solution turned into a visible blue product. The image of the reaction product captured using a digital camera was used without further modification. Finally, 20 μL H_2SO_4 (2 M) was added to each well to

Table 1
The sequence used in this assay. (The sequences used are as follows (5′-3′)).

Name	Abbreviation	Sequence (5′-3′)
Capture probe	CP	Biotin-TTTTTT**ACCCCTATCAC**
miRNA-155	miR-155	UUAAUGCUAAUCGUGAUAGGGGU
Reporter probe	RP	**GATTAGCATTAA**AGTCTAGGATTCGGCGTGGGTTAA
Hairpin1-FITC	H1-FITC	FITC—TTTTTTTTAACCCACGCCGAATCCTAGACT***CAAAGT***AGTCTAGGATTCGGCGTG
Hairpin2-FITC	H2-FITC	AGTCTAGGATTCGGCGTG***GGTTAA***CACGCCGAATCCTAGACTACTTTGTTTTTT—FITC
Hairpin 1	H1	TTAACCCACGCCGAATCCTAGACT***CAAAGT***AGTCTAGGATTCGGCGTG
Hairpin 2	H2	AGTCTAGGATTCGGCGTG***GGTTAA***CACGCCGAATCCTAGACTACTTTG

*In the hairpin sequences, loops are italicized, and sticky ends are underlined; in the CP and RP, the parts that are complementary to the miR-155 are bold.

convert the blue product into yellow, which was estimated at 450 nm using microplate reader (Infinite F500, Tecan, Sweden).

Furthermore, HCR-HRP assay was also carried out in fetal bovine serum to verify its practical performance. MiR-155 was diluted to a series of different concentrations in 100% fetal bovine serum. Other detection steps were as the same as those in buffer solution. Every experiment was repeated three times.

Results and discussion

Principle of miRNA detection by HCR-HRP assay

The sensing strategy was illustrated in Fig. 1. Two hairpin structures with potential energy, H1-FITC and H2-FITC were the key factors of the HCR reaction. In the absence of an initiator sequence, the mixture of H1-FITC and H2-FITC maintained a sub-steady state at RT, the two hairpin structures were not opened, and the hybridization reaction did not occur. When the RP (the blue area is the HCR startup sequence, pink area is target specific) was added to the hairpin structure mixture (H1-FITC and H2-FITC), it first hybridized with the sticky end of H1-FITC, gradually opened the whole hairpin structure through strand replacement reaction and simultaneously released the single strand, which acted as the initiator sequence for H2-FITC. After a few cycles, a conventional long double-helix polymer labeled FITCs was formed, called as "RP-HCR." In this study, the CP, labeled with biotin, was first immobilized on SA-coated plates through interaction between biotin and SA. Then the target miR-155 and RP-HCR were successively added to the plate, a sandwich-style "CP/miRNA/RP-HCR" complex was formed through base complementary, and fixed on the microplate. HRP-Ab continued to react with "CP/miRNA/RP-HCR" complex forming a double helix tag FITC combination, and was indirectly fixed to the surface of the microplate. This indicated that every target miRNA could introduce numerous HRP bindings to the plate, which enabled the TMB substrate turning from colorless to blue. After acidification, the quantitative data can be achieved at 450 nm by microplate reader. Conversely, when the target miR-155 didn't exist, the sandwich compound (the sandwich complex) "CP/miRNA/RP-HCR" couldn't be formed to be fixed to the plate and couldn't capture the probe FITC and HRP.

The best RP concentration for starting HCR

Several studies have shown that the average molecular weight of the polymers resulting from HCR was inversely correlated with the concentration of initiator. The experimental results were shown in Fig. 2(A). When the concentration of RP is 0 (lane 7), HCR should not be performed. However, little polymer continues to exist in the lane. This phenomenon may be explained that not all probes can form their ideal hairpin structure in the process of hairpin formation. There may be few probes remaining single strand, which can play the role of initiator sequence and trigger the HCR. However, any false positive results in the detection of target DNA were not observed despite little polymer remaining as analyzed by the gel electrophoresis. With increased RP concentration, the efficiency of HCR gradually improved, and the amount of the hairpin probe remaining gradually reduced. When 0.2 μM was reached (lane 5), the residual quantity of hairpin probe was minimum, and smears of HCR products with high molecular weight were detected. However, when the RP concentrations continued to increase, the polymer became scattered in length, which was not conducive to the final analysis. Thus, 0.2 μM for RP was considered adequate. Subsequent experiments adopted this most suitable concentration.

The best ratio of hairpin-FITC/hairpin

In another study, we found that the ratio of labeled hairpin/hairpin had a particular impact on HCR efficiency and the test performance because of a steric effect [22]. In this study, we also explored the best ratio of labeled and no labeled probes. As shown in Fig. 2(B), when the ratio of H1-FITC/H1 (H2-FITC/H2) was 9:1, the highest signal value was obtained. (The target was 10 pM). Thus, 9:1

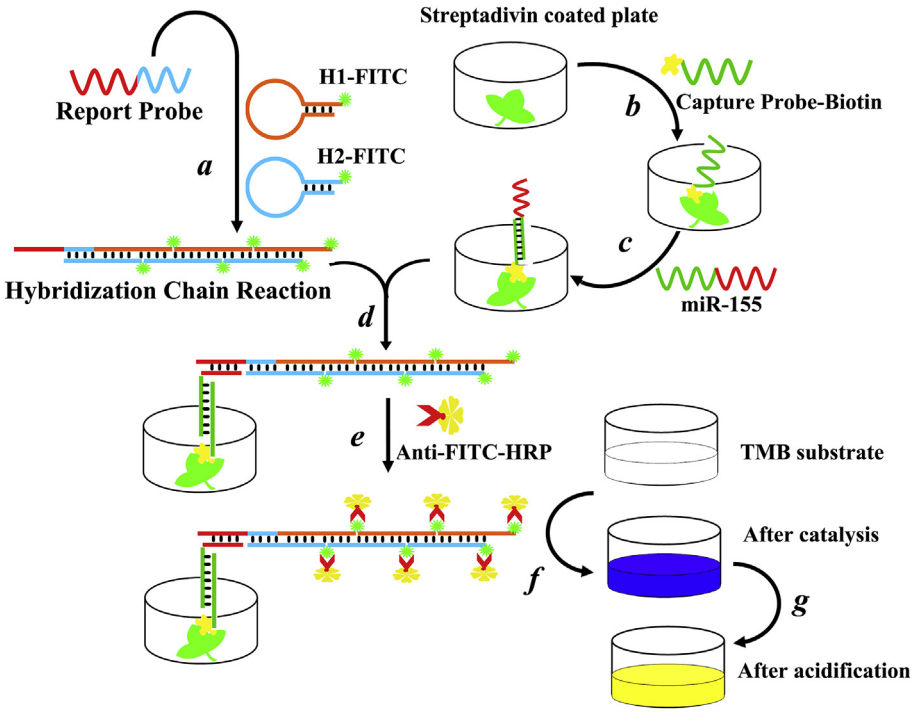

Fig. 1. Schematic representation of the sensing strategy for colorimetric detection of miR-155 based on HCR-HRP.

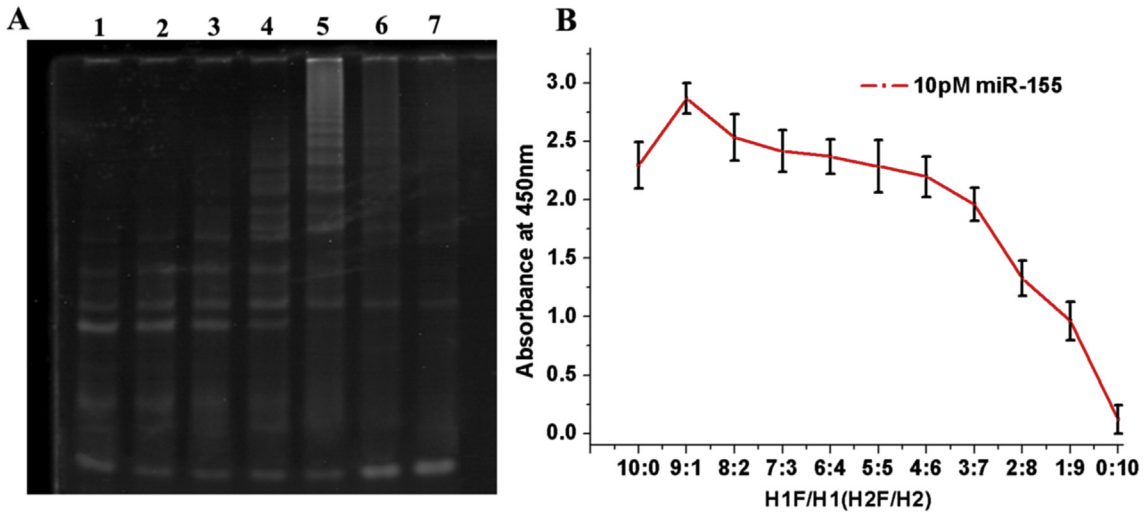

Fig. 2. **(A)** Effect of RP concentration on HCR amplification. Lanes 1–7: seven different concentrations of RP (1.0, 0.5, 0.4, 0.3, 0.2, 0.1 and 1.0 μM) in a mixture of H1-FITC (1 μM) and H2-FITC (1 μM) respectively. **(B)** Effect of the ratio of labeled hairpins vs hairpins on the detection performance. Calibration curve represent A450 corresponding to eleven different volume ratios of H1F/H1 (H2F/H2) when the target was 10 pM and RP was 0.2 μM.

was selected as the best ratio and used in the subsequent studies.

The best concentration and the optimal incubation time of CP

To assess the optimal coverage for hybridization, the amount of CP versus absorbance was selected and was shown in Fig. 3(A). As the amount of capture probe increased, signal value increased initially and reached a maximum at 0.5 μM of CP, then maintained constant with increasing CP concentration. These results suggested that an insufficient amount of capture probe affected the binding efficiency of CP/miRNA/RP-HCR on the microplate. Excessive free capture probe may result in waste of reagent. Thus, the concentration of CP (0.5 μM) was used in subsequent studies.

The effects of CP incubation time were shown in Fig. 3(B). Considering the efficiency and the cost, 1 h was selected and used in the subsequent studies.

Analytical performance of the miRNA detection

All the analytical experiments were performed under optimal conditions and the fabricated HCR-HRP biosensor showed great sensitivity in the detection of target miRNA in hybridization buffer and serum sample (Fig. 4(A)&4B). The logarithmic plots of miR-155 concentration vs. A450 were linear in the wide range from 1 pM to 100 pM (Fig. 4(C)). The calculated LOD was 31.8 fM in buffer solution and 100 fM in serum according to the rule of three times of standard deviation. Linear regression analysis of detection data yielded the following equation: A450 = 1.01 + 0.45 logC in buffer solution and A450 = 0.62 + 0.33 logC in serum, where C is the concentration of miR-155 in pM.

In this assay, HRP is the key factor for signal amplification. Its catalysis not only amplified the signal but also made the detection results visually observable. HCR also played an important role in signal amplification and its signal magnification ranged from 0 to 3 orders of magnitude compared the assay without HCR [23–26]. In our previous study, HCR for signal amplification showed a sensitivity of 176-fold higher than of the detection without HCR [22]. In this method, the assembly of the hairpin probes in HCR introduced the HRP, meaning the dual signal amplification. And the comparison in Tab S1&S2 revealed the high performance of the HCR-HRP

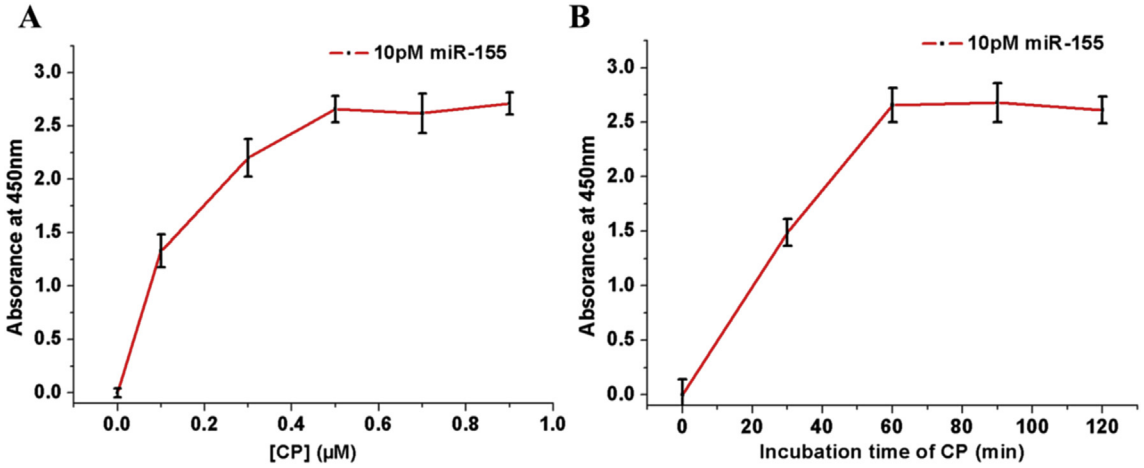

Fig. 3. Effect of CP on the performance of HCR-HRP assay. Test results when (A) seven different concentrations (0, 0.1, 0.3, 0.5, 0.7 and 0.9 μM) of CP and (B) different incubation time (0, 30 min, 60 min and 90 min) for CP were applied. The target was 10 pM.

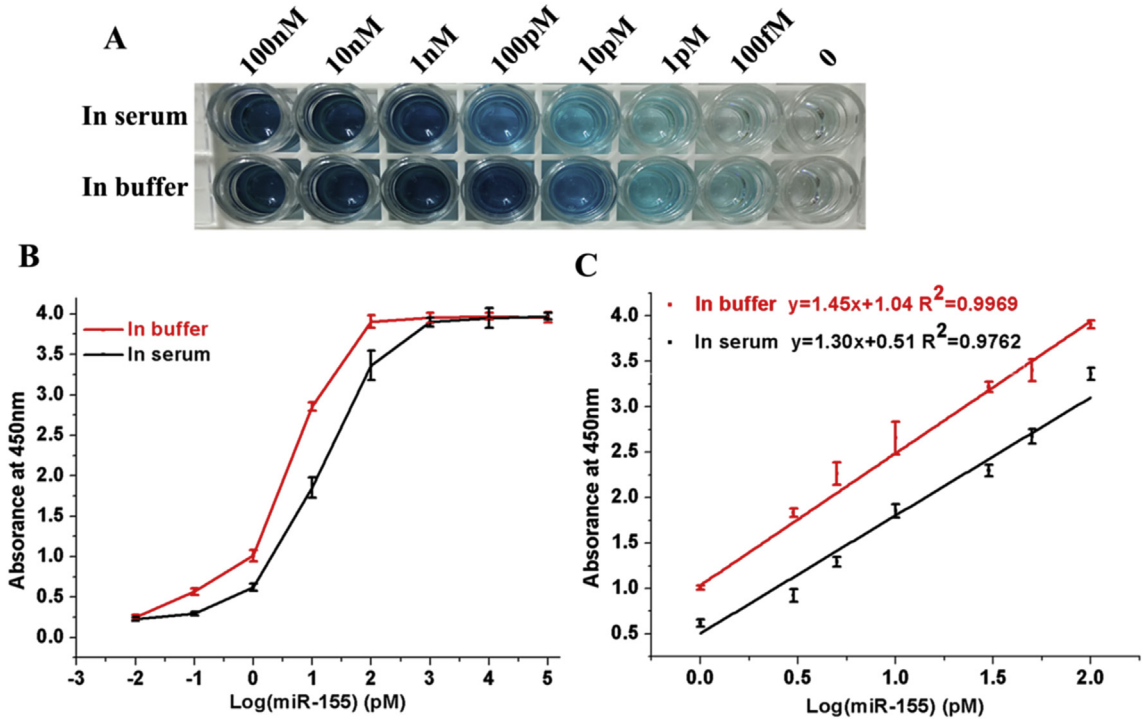

Fig. 4. Sensitivity assay for detection of miR-155 (0–100 nM) in buffer solution and in serum. (A) Photographic images of the assay performance. (B) Calibration plot of the absorbance at 450 nm (A450) vs log [miR-155]. (C) Linear part of the plot in (B), with the concentration of miR-155 ranging from 1 to 100 pM. Error bars represent the standard deviation of three independent measurements, which were all performed under the best experiment conditions.

assay. Besides, HCR was triggered by a certain amount of RP, instead of unknown quantity of target nucleic acid, meaning that the length of products (signal magnification) was uniform, not changing with the amount of the target. This may explained the wide linear range from 1pM to 100pM (100 amol to 10 fmol).

For the analytical performance of miR-155 in serum, only the target miR-155 was diluted with fetal bovine serum and other reagents were diluted with SPSC buffer. After the addition of target RNA, redundant miR-155 and the complex matrix such as proteins were washed away, not affecting the following procedure. Although the data shown in Fig. 4(B) revealed that the color signal of serum was slightly lower than that of buffer. This may because the small residual matrix competitively inhibited the combination of anti-FITC-HRP. In general, the detection results in serum sample also compared well with those of buffer solution, which clearly demonstrated this miRNA detection biosensor had potential for analytical application in detection of miRNAs in real biological samples.

Specificity of the assay

Another important aspect of the performance of the HCR-HRP assay is its selectivity towards the detection of the target without interfering signals from non-specific interactions. The specificity was examined by measuring a target sequence and single-base, two bases, three bases, and four bases mismatched sequences. In the HCR-HRP assay, the perfectly matched sequence produced the most robust signal, which was three or more fold stronger than that observed from the other mismatched miRNA sequences. The experimental errors were mainly caused by residual quantity of the un-reacted anti-FITC-HRP and the slight delay of the addition of the stop buffer. These results indicated that the HCR-HRP detection platform exhibited an excellent capability in differentiating even a single-base mismatch with the target sequence, which demonstrated the appropriate selectivity of this platform. This is relevant for diagnosing diseases related with miRNA. Therefore, the proposed technique is particularly attractive for the detection of specific nucleotide sequences (Fig. 5).

Conclusions

In conclusion, hybridization chain reaction in conjunction with HRP for signal amplification and signal readout on microwell plates offers a versatile platform for rapid, sensitive, and practical detection of microRNA. First, the whole process of detection can be

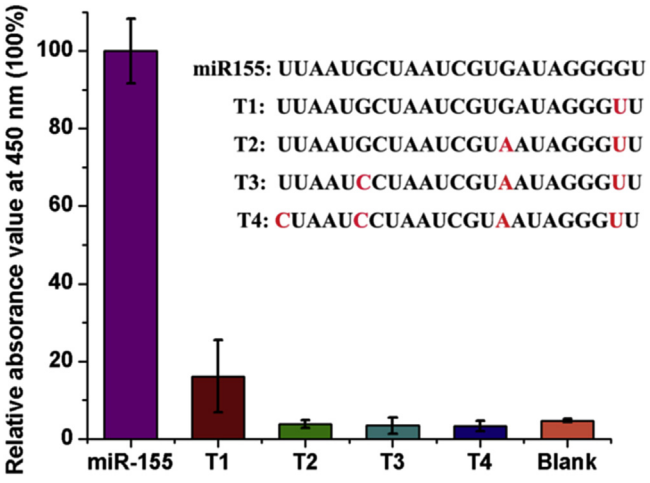

Fig. 5. The selectivity of HCR-HRP assays. Histograms represent target miR-155, single-, two-, three-, four bases mismatched sequences (10 pM) and negative control. Error bars represent the standard deviation from three independent experiments.

accomplished in 4 h because of its simplified steps. Second, the HCR-HRP assay enables the visual detection of miRNAs with a LOD of 31.8 fM. Moreover, this protocol was successfully carried out in the fetal bovine serum with a good matching rate and a LOD of 100 fM. Thus, this system is promising for application in biological research and clinical diagnostics.

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Abbreviations

MiRNAs	MicroRNAs
HRP	horseradish peroxidases
HCR	hybridization chain reaction
FITC	fluorescein isothiocyanates
miR-155	miRNA-155
RT-qPCR	quantitative real-time PCR
SPR	surface plasmon resonance
RT	room temperature
HCR-HRP	hybridization chain reaction in conjunction with horseradish peroxidase
anti-FITC-HRP	anti-FITC monoclonal antibody-HRP
LOD	limit of detection
SPSC	sodium phosphate sodium chloride
SA	Streptavidin
TMB	4,4′-diamino-3,3′,5,5′-tetramethylbenzidine
CP	capture probe
RP	reporter probe

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.04.007.

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