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Original Research Article

Duplex microRNAs assay based on target-triggered universal reporter hybridization

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ABSTRACT

In this paper, we designed and evaluated a duplex detection strategy for microRNAs (miRNAs) using universal probe-based target-triggered double hybridization and fluorescent microsphere-based assay system (xMAP array). In the absence of target miRNA, reporter DNA cannot hybridize stably with the immobilized capture DNA due to its low melting temperature. Only after adding target miRNA, can reporter probe hybridize with capture probe to form a stable three-component complex. This target-triggered stable hybridization makes this method possible for highly selective and sensitive detection of multiple miRNAs. We exemplified a quantitative detection of duplex miRNAs with a limit of detection of 40 pM. The xMAP array platform holds the potential of extending this approach to simultaneous detection of up to 100 miRNA targets. Considering the simplicity, rapidity and multiplexing, this work promised a potential detection of multiple miRNA biomarkers for early disease diagnosis and prognosis.

1. Introduction

More than 2500 microRNAs (miRNAs) have been found in humans, and most of them play important regulatory roles in a wide range of biological processes, including cellular differentiation [1], proliferation, apoptosis [2] and development [3]. Abundant studies have revealed that miRNAs are associated with various human diseases, including cancers such as breast cancer, ovarian cancer, and lung cancer [4–6], cardiovascular disease [7,8], neurological disease [9], and diabetes [10,11]. An ideal diagnostic miRNA assay should be sensitive, specific, simple, rapid, and multiplexed. However, high sensitivity and high specificity of miRNA detection are challenging because of the unique characteristics of miRNA such as the short length of miRNA sequences, the sequence homology among family members, and low abundance [12,13]. Current standard miRNA detection strategies include Northern blot, microarray, and reverse-transcription polymerase chain reaction, which generally require large amounts of sample and target amplification, and are labor-intensive, and not easily multiplexed [14,15].

The three-component strategy, which involves a pair of DNA probes (capture and labeled reporter probes) that flank the target sequence, has been widely employed in the DNA and RNA detection assays. However, the short length of miRNAs makes it difficult to form a strong and specific three-component complex. For example, Qiu et al. [16] and Díaz et al. [17] developed Tb-to-QD FRET-based hybridization assays and found that hybridization of both donor and acceptor probes to a single short miRNA strand is not very stable at low concentrations. Arata et al. [18] developed a method for miRNA detection by sandwich hybridization taking advantage of the coaxial stacking effect. They found that the efficiency of hybridization between capture and miRNA is the limiting factor in the sandwich hybridization. To overcome this issue, the direct detection of miRNA was made through the use of fluorescent locked nucleic acid (LNA) probes, whose higher binding affinity to miRNA compared with conventional DNA enabled the use of short probe lengths [19]. Sometimes, probe ligation is used to stabilize the miRNA to the capture [20,21]. In this regard, our group in 2010 reported a new strategy of template-dependent hybridization for short-length DNA detection based on target-enabled hybridization of a capture probe with a long reporter probe [22]. In 2012, we further employed a template-dependent surface hybridization using quantum dot-enhanced methodology to detect dual short RNA sequences [23]. A multiplexed "mix-and-measure" method would be highly desirable for measuring the expression levels of miRNAs. However, in the template-dependent hybridization assay, any permutation of eight-nucleotide tail sequence of the long reporter probe is limited, which causes limit to the simultaneous detection of multiple miRNAs.

The Luminex xMAP array is a microsphere-based multiplex system which can simultaneously detect up to 100 analytes from a single sample. Herein we developed a novel universal probe-based target-triggered double hybridization strategy for the detection of dual miRNAs utilizing xMAP array platform. To achieve this purpose, the corresponding capture probes were immobilized on a specific

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color-coded microsphere. Each capture probe was designed to have three functional segments, namely 5' poly(A) spacer sequence, middle complementary sequences to specific miRNA, and 3' complementary sequence to a biotin-modified eight nucleotide reporter probe. In the absence of specific target, universal reporter probes and capture probes cannot anneal to each other due to low melting temperature. Only after target miRNA bound to capture recognition section, reporter DNA was allowed to hybridize with capture probe. Thus the biotin labeled on the reporter probe was attached to the microsphere surface, and then reacted with streptavidin-phycoerythrin (SA-PE) to emit fluorescence. Although a duplex analysis is demonstrated here, higher plexing is possible. The proposed method possesses practical adaptability and generality for higher plexing miRNA detection. Only the recognition domain of capture probes needs to be designed according to target sequences, which simplifies the design of higher plexing miRNA detection. The utilization of universal reporter probe in simultaneous assay of multiple miRNAs can also alleviate the cross interference between multiple capture and reporter probes. In view of these advantages, the strategy developed here provides a universal technology for developing simple biosensors for sensitive and selective detection of multiple miRNAs.

2. Materials and methods

2.1. Materials and reagents

All oligonucleotides were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China), and miRNAs were synthesized by Takara Biotechnology Co., Ltd (Dalian, China). The sequences of oligonucleotides used in this work are listed in Table 1. Carboxylated microspheres were purchased from Luminex Corp. (Austin, TX, USA). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) was obtained from Sigma-Aldrich (Shanghai, China). Bovine serum albumin (BSA) was provided by Aizite Biotechnology Corp. (Shanghai, China). SA-PE was purchased from Jackson ImmunoResearch (West Grove, PA). Sheath fluid was procured from Bio-Rad Corp. (Shanghai, China). MiRNeasy mini kit was purchased from QIAGEN (Venlo, Netherlands). All chemicals were of analytical grade and were used as received. All of the solutions were prepared with ultrapure water from a Millipore Milli-XQ system (Bedford, MA, USA), treated with DEPC.

2.2. Preparation of capture DNA-microspheres conjugates

Before conjugation, carboxylated microspheres were activated in 5 mg/mL EDC and 0.2 M imidazole buffer (pH 6.0) for 20 min at 37 °C with shaking. Then capture probes were conjugated with activated carboxylated microspheres in 0.2 M imidazole buffer for 2 h at 37 °C with shaking. The reaction volume was proportional to the amount of microsphere. The probe-conjugated microspheres were washed once with wash buffer (7 mM Tris-HCl, pH 8.0, 0.17 M NaCl, and 0.05% Tween 20), and then blocked in buffer A containing 10% BSA for 1 h. After that, the supernatant was aspirated and discarded, and the coupled microspheres were resuspended in appropriate volume buffer A (20 mM Tris-HCl, pH 8.0, and 0.5 M NaCl) to bring the microsphere density at the same level as the stock material before conjugation. The capture probe-conjugated microspheres can be stored at 4 °C for a few days before use. For multiplex assay, each type of microspheres was individually conjugated with a specific capture DNA before being pooled together for the remaining of the procedure. In this work, two products of Magplex® microsphere coded 35 and 53 were used.

2.3. Target-triggered universal probe hybridization strategy for the detection of multiple miRNAs

Depending on experiment scale, an appropriate amount of capture probe-conjugated microspheres was removed from storage and transferred to 96-well plates in the amount of 0.5 μL stock/well. After magnetic separation, the supernatant was discarded. Mixture of miRNA targets and reporter probes prepared in designated concentration were added into each well (25 μL/well, in buffer A), and incubated at the designated temperature for 1 h. In a duplex assay, the two microsphere types were pooled at an equal volume before dispensing to the 96-well plate. After that, the microspheres were washed once with 50 μL wash buffer. The detection step was performed in 50 μL/well of SA-PE prepared in buffer A containing 1% BSA. After 30 min of incubation, the microspheres were washed once in 100 μL wash buffer, and then re-suspended in 100 μL of sheath fluid for analyzing on the Luminex 200 instrument.

2.4. Cell culture and total RNA extract measurement

Human breast cancer cells (MCF-7) were cultured according to the instructions of the American Type Culture Collection. Cells were grown in DMEM (Gibco, penicillin 100 U/mL, streptomycin 100 μg/mL) plus 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were collected and centrifuged at 3000 rpm for 5 min in a culture medium, and then washed once with PBS buffer. Total RNA samples were extracted using the QIAGEN miRNeasy mini kit. The extracted RNA was eluted into approximately 50 μL of H_2O. The total RNA concentration was determined by the UV absorption at 260 nm with the following formula: concentration of RNA sample = 40 μg/mL × A260 × dilution factor. The RNA sample in these cells was diluted to 1 μg/μL before analysis. The following procedure of miRNA detection was as described in the target-triggered universal reporter hybridization.

Table 1
DNA and miRNA sequence used in this work.

Name	Sequence (5'-3')
Capture-miR-21	5'-NH_2-AAAAATCAACATCAGTCTGATAAGCTA**TCAACACC**-3'
Capture-miR-31	5'-NH_2-AAAAAAGCTATGCCAGCATCTTGCCT**TCAACACC**-3'
miR-21	5'-UAGCUUAUCAGACUGAUGUUGA-3'
miR-31	5'-AGGCAAGAUGCUGGCAUAGCU-3'
Universal-reporter-6	5'- biotin-AAAAATGTTGA-3'
Universal-reporter-7	5'- biotin-AAAAAGTGTTGA-3'
Universal-reporter-8	5'- biotin-AAAAAGGTGTTGA-3'
Universal-reporter-10	5'- biotin-AAAAAGTGGTGTTGA-3'
Capture-miR-21-gap	5'- NH_2-AAAAATCAACATCAGTCTGATAAGCTA**TCAACACCAC**-3'
Universal-reporter-gap1	5'- biotin-AAAAATGGTGTTG-3'
Universal-reporter-gap2	5'- biotin-AAAAAGTGGTGTT-3'

The underlined section was complementary with target sequences. The boldface section was complementary with reporter probes.