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Short communication

Direct microRNA detection with universal tagged probe and time-resolved fluorescence technology

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ABSTRACT

microRNAs have emerged as the central player in gene expression regulation and have been considered as potent cancer biomarkers for early disease diagnosis. Direct microRNA detection without amplification and labeling is highly desired. Here we present a rapid, sensitive and selective microRNA detection method based on the base stacking hybridization coupling with time-resolved fluorescence technology. Other than planar microarrays, magnetic beads are used as reaction platforms. In this method, one universal tag is used to report all microRNA targets. Its specificity allows for discrimination between microRNAs differing by a single nucleotide, and between precursor and mature microRNAs. This method also provides a high sensitivity down to 20 fM. Moreover, the full protocol can be completed in about 3 h starting from total RNA.

1. Introduction

microRNAs (miRNAs) are a class of small RNA molecules (~22 mers) that bind to complementary sequences in the 3′-untranslated region of mRNAs (Bartel, 2004). These tiny miRNA molecules found in plants, humans, and animals perform key functions in gene silencing and affect early-stage cell development, cell differentiation, and cell death (Hwang and Mendell, 2006). Moreover, recent studies have pointed towards a connection between specific changes in miRNA expression and various diseases, especially for cancers (Arenz, 2006; Lu et al., 2005; Bartels and Tsongalis, 2009; De Martino et al., 2009). From these findings, it has also been established that miRNA expression profiling is a basic and preliminary procedure in most miRNA studies.

So far, many methods have been developed for miRNA profiling, including northern blotting, Real-Time PCR, sequencing and microarray (Cissell and Deo, 2009). Different from mRNAs, miRNAs show some unique features, such as small size, lacking of 5′poly(A) tail, and homology between miRNA species, etc. Consequently, direct detection of miRNA without labeling or amplification is highly desired for the potential diagnostic applications. Novel efforts have been proposed to achieve this target using non-labeling strategies, including electrochemical devices (Sprinzl and Poehlmann, 2010), molecular beacons (Paiboonskuwong and Kato, 2006; Kato, 2008; Hartig et al., 2004), nanopore sensors (Wanunu et al., 2010), atomic force microscopy (AFM) (Husale et al., 2009), surface plasmon resonance (SPR) (Fang et al., 2006) and so on.

Herein, we describe a simple label-free miRNA detection method by using a universal tag (UT) to report all miRNA targets. Being superior to conventional sandwich hybridization strategy (Barad et al., 2004), we can use only one report probe (named UT) to realize multiplex detection. This minimizes labeling bias over different miRNA targets and lowers the analyzing cost. The UT can be detected by time-resolved fluorescence (TRF) technology for transforming hybridization events into fluorescence signal and read out with a plate reader. Rather than planar microarrays, we choose magnetic beads as reaction platforms which provide faster solution-phase kinetics.

2. Experimental

2.1. DNA and RNA oligonucleotides

All oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. and Bioneer Corporation (Korea).

Their sequences are listed in Supplementary information (Table S1–S3).

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2.2. Preparation of capture probe-conjugated magnetic beads

The capture probe-conjugated magnetic beads were prepared by conjugating the carboxylated magnetic beads with the 5′-NH_2 modified capture probe according to the manufacturer's instructions. Specifically, 2×10^6 carboxylated magnetic beads (2.8 μm, Dynabeads® M-270 Carboxylic Acid, Invitrogen Corporation) were pipetted into a microtube and washed twice with the equal volume of MES buffer (100 mM, pH 4.5) (Alfa-Aesar). The magnetic beads were resuspended in 50 μL MES buffer. Then 2 μL amino-substituted capture probe (100 μM) was added for the coupling reaction. The coupling reaction was initiated by adding 2.5 μL of freshly prepared 10 mg/mL EDC (Alfa-Aesar). The mixture of magnetic beads, capture probes, and EDC was incubated at room temperature for 30 min. This step was repeated for a total of four times. After the coupling reaction, the conjugated magnetic beads were washed with 0.02% Tween and 0.1% SDS. In the end, the capture probe-conjugated magnetic beads were resuspended in MES buffer and stored at 4 °C.

2.3. miRNA hybridization reactions

For miRNA hybridization, miRNA targets at various concentrations and 50 nM biotinylated UT (final concentration) were added to hybridization buffer (5 × SSC/0.2% SDS). Capture probe-conjugated magnetic beads (~5000 beads) were also added to the hybridization buffer. The final hybridization volume was 100 μL for each reaction. Then the hybridization solution was incubated at 42 °C for 1 h in a microtube incubator with a shaking speed of 300 rpm. After hybridization, the hybridized magnetic beads were washed twice with 2 × SSC/0.1% SDS at 42 °C, then twice with 0.2 × SSC at room temperature.

2.4. Fluorescence signal detection through time-resolved fluorescence technology

Eu-labeled Streptavidin (PerkinElmer) was diluted to 100 ng/mL in assay buffer (containing Tris–HCl, NaCl, BSA, Tween 20, EDTA and NaN_3) within 1 h of use. Then 100 μL of the immunoassay solution was added to resuspend the hybridized magnetic beads. After 1 h incubation at room temperature with slowly shaking, the beads were concentrated using a magnet and the supernatant was discarded. The beads were washed six times using 1 mL of washing buffer (containing Tris–HCl, NaCl, Tween 20 and NaN_3, pH 7.8) for 2 min each time at room temperature. A 50 μL of Enhancement Solutin (Cat# 1244-104, PerkinElmer) was added to resuspend the hybridized beads and the mixture was shaken slowly for 5 min at room temperature. Then the mixture was transferred to a Costar black microtiter plate and read in time-resolved fluorescence instrument Victor^2 (PerkinElmerWallac, Gaithersburg, MD) at excitation and emission wavelength of 340 and 615 nm, respectively.

3. Results and discussion

3.1. Direct miRNA detection strategy

Our system for miRNA detection relies on a sandwich-like assay comprising a capture probe immobilized on magnetic bead, a 20-mer UT linked with biotin, and a miRNA target (Scheme 1). The capture probe has three sequence segments, the central segment complementary to miRNA target, an identical 8-mer tail complementary to the biotinylated UT and a poly$(A)_{10}$ as a spacer. In this methodology, miRNAs are directly applied for hybridization together with the biotinylated UT. When a sample solution is introduced, both the miRNA target and the UT hybridize with the capture probe. However, only the longer duplex between miRNA and capture probe is energetically favorable under hybridization condition, which can provide additional stability to the short duplex between UT and capture probe. This phenomenon, called base stacking hybridization, has been used to describe the additional stability associated with DNA hybridization reactions wherein two or more DNA oligonucleotides hybridize in a contiguous tandem arrangement to a longer complementary DNA single strand (Parinov et al., 1996; Vasiliskov et al., 2001; O'Meara et al., 1998). After hybridization is completed, the fluorescence signal is measured through TRF technology. TRF is a sensitive nonradioactive detection technique which involves the use of lanthanide chelates as fluorescent labels. Due to the unique fluorescence properties of lanthanide chelates, such as the long decay time, large Stokes' shift, high fluorescence intensity, the TRF technology possesses low background fluorescence, high sensitivity and wide dynamic range of measurement compared to conventional fluorescence technologies (Soini and Lovgren, 1987). Until now, this technology has been successfully utilized for chemical and biological assays, particularly using europium chelates as probes (Evangelista et al., 1988; Harma et al., 2000; Hagan and Zuchner, 2011).

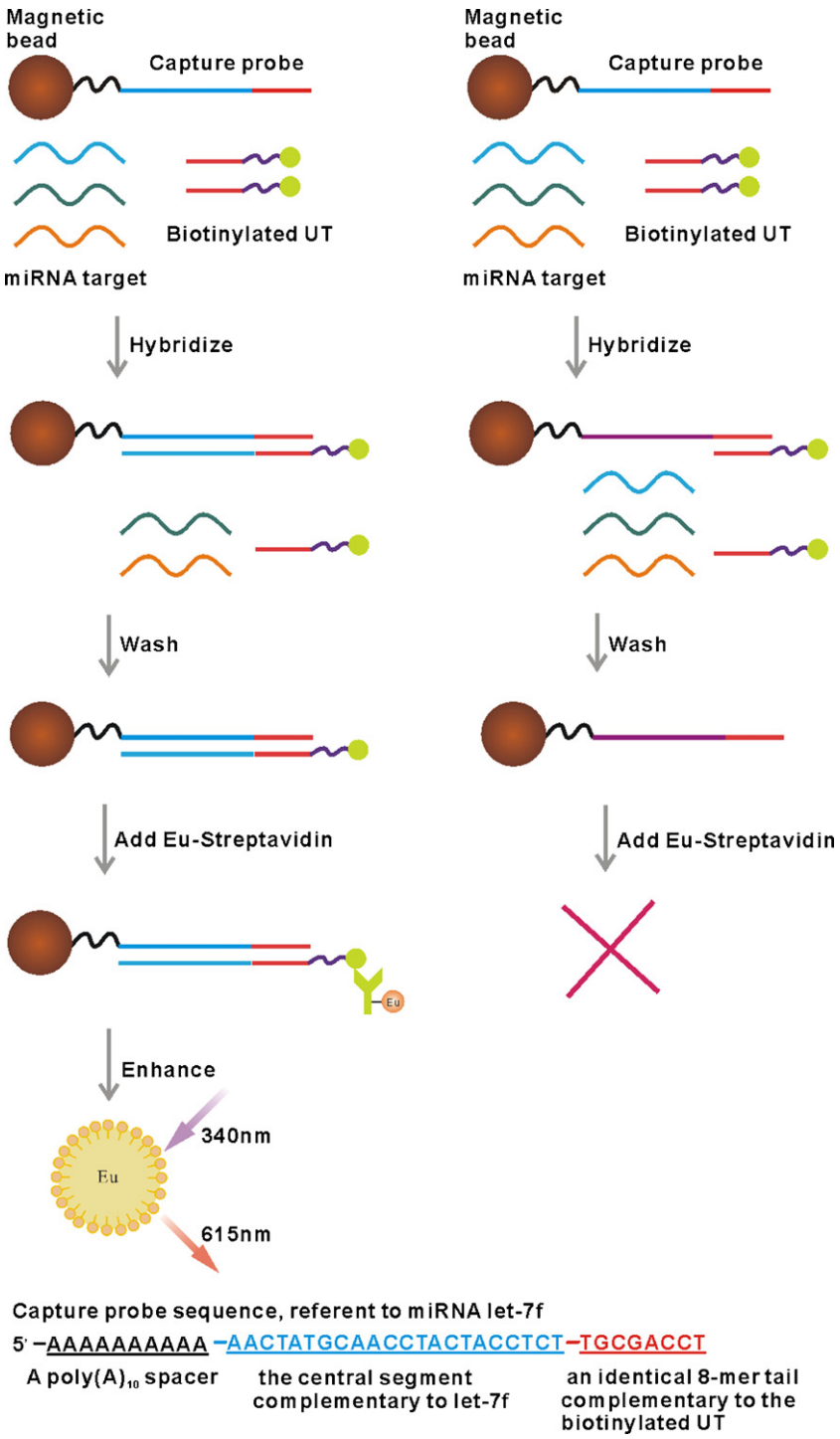
3.2. Specificity

A distinctive nature of miRNAs is the highly sequence similarity between miRNA family members, which may pose extra challenge for any miRNA assays. To examine the specificity of our assay, 200 pM of four synthetic let-7 miRNA family members, let-7a, let-7b, let-7d and let-7f (Reinhart et al., 2000; Pasquinelli et al., 2000) were hybridized with capture probe P-let-7f individually. Details of the oligonucleotides sequences are listed in Supplementary information. Having only a single base mismatch between let-7a and let-7f, they show 24.7% cross-hybridization (Fig. 1a). Secondly, the cross-hybridization between let-7d and let-7f is lower than that between let-7b and let-7f (both having three base mismatches). We propose that this is a direct consequence of the base stacking effects: any mismatch positioned at the stacking site may seriously weaken the stacking interactions. In fact, Mirzabekov's group described the similar results, that even a single base mismatch in stacking site would effectively hinder the stacking interactions (Vasiliskov et al., 2001). These results clearly imply that our method demonstrates high specificity. The cross-hybridization can be further reduced via probe designing optimization.

Precursor miRNA (pre-miRNA) is a stable hairpin containing an entire mature miRNA sequence, which may interfere with mature miRNA detection (Lee et al., 2003). A purification procedure to eliminate signals from pre-miRNAs is usually required before miRNA analysis (Lu et al., 2005; Cissell et al., 2007; Shingara et al., 2005), whereas our strategy requires no such separation and can deal directly with total RNA. To evaluate this capability, human miR-31 and its precursor pre-miR-31 were hybridized with probe P-miR-31. From the results, the signal generated by pre-miR-31 is only about 10% of miR-31 (Fig. 1b), demonstrating that our assay can differentiate between mature miRNAs and their precursor forms easily.

3.3. Sensitivity

Another intriguing feature of our assay is its high sensitivity, which is crucial for sensitive miRNA detection, because miRNAs represent only a small fraction (ca. 0.01%) of the mass of a total RNA sample (Bartel, 2004). To determine this performance, we conducted hybridizations with let-7d as target ranging in final concentration from 200 pM down to 20 fM. The minimum amount of let-7d detected is 2 amol (a 20 fM concentration with a total volume of 100 μL) (Fig. 2). This detection limit of 20 fM is significantly



Scheme 1. Schematic showing the detection of microRNAs using a combination of base-stacking hybridization and TRF technology. The capture probe contains three segments, the central segment complementary to miRNA targets, an identical 8-mer tail complementary to the biotinylated UT and a poly(A)10 as a spacer. The UT is captured only in the presence of target miRNA. Then the signal can be detected through TRF technology. The right diagram illustrates that there is no perfectly matched miRNA target.

lower than most of previously reported non-amplification methods (Yin et al., 2008). The solution based detection, high efficient labeling and TRF technique are believed to contribute a lot to this performance.

3.4. *miRNA detection using total RNA from cell lines*

Some previous reports showed that miRNAs had different expression levels between the normal lung fibroblast cell line HLF and the lung carcinoma cell line A549 (Volinia et al., 2006; Takamizawa et al., 2004). To demonstrate the performance of our method for real biological samples, we measured two miRNAs, miR-21 and let-7a from total RNA extracted from the two cell lines. In this experiment, 1.0 μg total RNA was applied for hybridization directly. Our results indicate that miR-21 is remarkably overexpressed in A549, while let-7a shows reduced expression level in A549. These results clearly correlate with those previous reports, and also are in accordance with results from our quantitative

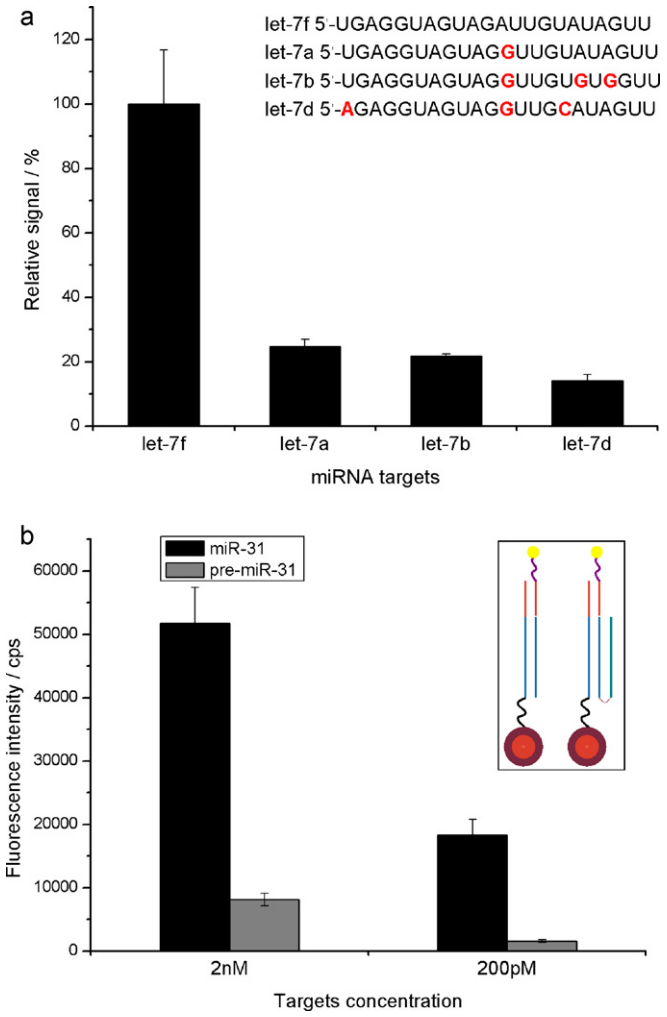


Fig. 1. The specificity evaluation for the bead-based miRNA detection. (a) 200 pM of four synthetic let-7 miRNA family members were hybridized with capture probe P-let-7f individually. The red bold letters represent the mismatched bases. The fluorescence intensity of the targets were reported as the percentages of the intensity of target let-7f. (b) The bead-based miRNA detection assay is specific in discriminating mature miRNA and precursor miRNA. 2 nM and 200 pM of miR-31 and pre-miR-31 were subjected to the assay respectively.

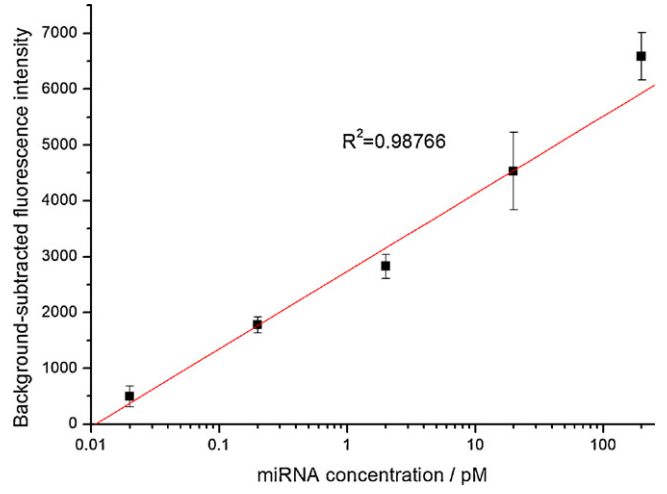


Fig. 2. Sensitivity and dynamic range of the let-7d assay. Synthetic let-7d input ranged from 20 fM to 200 pM per reaction (final concentration).

real-time PCR to a certain extent. (See Supplementary information, Fig. A.1.) Without labeling and PCR amplification, it is possible that our method can be directly applied for whole blood miRNAs detection.

4. Conclusions

In summary, we have developed a simple bead-based hybridization-triggered signaling strategy for direct miRNAs detection from total RNA. The coupling of conventional hybridization based method and stacking hybridization based method endow this assay with the remarkable features such as rapid, label-free detection, single mutation and precursor miRNA discrimination and complex biological samples analysis. When compared with conventional magnetic bead base-methods, such as p19 miRNA detection kit (NEB), the analyzing cost is reduced because of the usage of universal tag in multiplex detection. Besides that, our system has excellent detection sensitivity, resulting from solution-phase kinetics, magnetic beads capturing and high signal-to-noise ratio features of TRF technology. This method, in conjunction with the 96-well or 384-well matrix should prove useful for medium-throughput expression profiling of miRNAs. Multiplex miRNA targets can also be analyzed in the same well by using different lanthanides TRF labels with distinct emission wavelengths under the same excitation wavelength. When applied in DNA microarray, our strategy has been suceeded in profiling high-throughput miRNA using total RNA from human tissues (Duan et al., 2011). Furthermore, for miRNAs with natural modifications at 3′ ends such as plant miRNAs (Yu et al., 2005), some methods, such as enzymatic ligation (Chapin et al., 2011) would be problematic. Without labeling or ligation, our method could be applied directly in plant miRNAs research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.01.035.

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