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Detecting miRNAs by liquid hybridization and color development

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ABSTRACT

Currently, two methods, PCR and Northern blot, are widely used to detect individual microRNAs (miRNA). Although PCR is highly sensitive, false positives and difficulties of primer design discourage its use. While a Northern blot is an effective tool, traditional Northern blot protocols are complicated, time-consuming, and usually inconvenient for users. Liquid Northern blot methods are rapid but require instruments for detection of fluorescent signals. Here, we describe an alternative protocol, liquid hybridization and color development (LHCD), based on the rapidity of liquid hybridization and the signal amplification of avidin–biotin complex (ABC) for detection. LHCD can distinguish a one-nucleotide difference within a miRNA family and allow for the sensitive detection of 2.5 f mol of miRNAs. Furthermore, LHCD is not only simple and rapid, but detection is visual and so it does not require expensive equipment. LHCD is easy to learn and convenient for miRNA analyses.

1. Introduction

MicroRNAs (miRNAs) are ~21-nucleotide RNAs in plants, animals and microbes. They have emerged as key post-transcriptional regulators of gene expression and have revolutionized our comprehension of gene expression [1,2]. Predictions suggest that about one-third of all protein-coding genes are regulated by miRNAs [3]. In mammals, miRNAs are predicted to control the activity of ~50% of all protein-coding genes, and are involved in the regulation of almost every cellular process investigated so far [2]. Importantly, their altered expression is associated with many human pathologies [2]. To date, release 18 of the miRBase sequence database contains 21,643 mature miRNAs from 168 species (http://www.mirbase.org/), and novel molecules are constantly discovered. However, the biological characterization and the functional confirmation of most miRNAs remain to be investigated.

Since the first miRNA gene was discovered in 1993 [4–6], great advances have been made in miRNA biology. However, the small size of miRNAs increases the technical difficulty of their identification, spatiotemporal detection, and functional confirmation. Generally, to investigate an individual miRNA, PCR and Northern blotting are used to measure miRNA expression levels in vitro. PCR is undoubtedly highly sensitive [7], however, the rates of false positives and difficulties designing primers limit its use. Northern blotting is the gold standard for directly examining the expression of miRNA [8]. Traditional Northern blot protocols include fractionating small RNAs by gel electrophoresis; transferring the separated RNA fragments onto a nylon membrane; over-night hybridization; and hours to days or even months of autoradiography [9,10]. The method is complex, lengthy, and inconvenient and in general, does not lend itself for simple and rapid analyses.

The new technology of liquid Northern hybridization overcomes these shortcomings and allows quick and simple detection of miRNA [11]. However, the use of fluorescent probes can present problems of decreased sensitivity due to fluorescence quenching. Furthermore, instruments for detection of fluorescent signals must be available. Avidin–biotin complex (ABC) method is a standard tool in immunohistochemistry (IHC). The avidin–biotin interaction is one of the strongest known non-covalent interactions ($K_d = 10^{-15}$ M) [12], making it ideal for both purification and detection strategies [13].

Based on our experience with Northern blotting, IHC, and Western blotting methods, we developed an alternative method of liquid hybridization and color development (LHCD), which combines the rapid features of liquid hybridization and the amplified signals provided by avidin–biotin complex detection [14,15].

Abbreviations: LHCD, liquid hybridization and color development; ABC, avidin–biotin complex; IHC, immunohistochemistry; AP, alkaline phosphatase; HRP, horseradish peroxidase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; DAB, 3,3′-diaminobenzidine; ECL Prime, Amersham™ ECL™ prime Western blotting reagent; SSWF, Super signal® west femto; BSA, bovine serum albumin; TBS, tris-buffered saline.

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We describe a LHCD protocol that provides an easy-to-learn and convenient-to-use tool for detecting miRNAs.

2. Method

2.1. Outline

Purified small RNAs are hybridized in buffer with 5′ biotin-labeled DNA probes. The hybridized mixture is dotted onto a nylon membrane after non-hybridized probes are digested with Exonuclease I. The membrane is then incubated with ABC. Finally, the membrane is developed with BCIP/NBP or DAB to produce colorimetric end products; ECL prime can be used to produce light for detection (Fig. 1).

2.2. Liquid hybridization

Hybridization buffer is pipeted into an Eppendorf tube. Synthesized or isolated small RNAs and 5′ biotinylated probes are added into the hybridization buffer, mixed thoroughly, and heated at 94 °C for 4 min. The hybridization reaction is performed at 42–65 °C for 60 min. Finally, digest non-hybridized probes with Exonuclease I at 37 °C for 30 min.

2.3. Color development

Dot the digested hybridization mixture onto a nylon membrane, dry, and perform ultraviolet crosslinking. Block the membrane with 10% BSA, then incubate the membrane with ABC–AP or ABC–HRP at 37 °C for 30 min. Wash the membrane with TBS buffer six times. Finally, develop with BCIP/NBP, or DAB, or ECL Prime.

2.4. Experimental protocol

2.4.1. Prepare total RNA sample

Isolate total RNAs by adding TRIzol (Invitrogen) to cells following the manufacturer's protocol.

2.4.2. Purify small RNAs (optional procedure)

Small RNAs are separated and purified from total RNAs by urea–PAGE (Urea–polyacrylamide gel electrophoresis) following protocols that are available from a number of laboratory websites (http://www.umassmed.edu/Content.aspx?id=154408&linkidentifier=id&itemid=154408; http://web.wi.mit.edu/bartel/pub/protocols.html). Alternatively, other methods, such as the FlashPAGE™ Fractionator System (Ambion, USA) can be used according to the manufacturer's instructions.

2.4.3. Prepare reagents and materials

Prepare hybridization buffer, 5′ biotinylated DNA probes, Exonuclease I, BSA, nylon membrane, ABC–AP or ABC–HRP, 1× TBS, and BCIP/NBT, DAB, ECL Prime, or SSWF. Hybridization buffer (Buffer A: 30 mmol/L Sodium phosphate buffer (pH 8.0), 0.3 mol/L of NaCl, 10 mmol/L of EDTA (11); or Buffer B: 1× Exonuclease I). 5′ biotinylated DNA probes are cheaper, and can be easily synthesized by commercial company (e.g., Takara, Japan). Exonuclease I, BSA, ABC–AP, ABC–HRP, 1× TBS, Triton-X-100, Tween-20, BCIP/NBT, and DAB are common inexpensive reagents readily available from commercial sources. Here, Exonuclease I was ordered from NEB (USA); BSA, Triton-X-100, Tween-20, ABC–AP, ABC–HRP, BCIP/NBT, and DAB from Boster (China), 1× TBS buffer from Dycent Biotech (China); ECL Prime from Amersham (USA). SSWF (Super Signal® West Femto) is expensive and is available from Thermo Scientific (USA). Nylon membrane is from Roche (Switzerland).

2.4.4. Liquid hybridization

Up to 16 μL hybridization buffer is loaded into a 200 μL Eppendorf tube. A specific amount of small RNAs (such as 20 pmol) and 5′ biotinylated probes (such as 20 pmol) is added into the hybridization buffer; mix thoroughly and heat the mixture to 94 °C for 4 min. Next, carry out the hybridization reaction by incubating the mixture in a water bath at 42–65 °C for 60 min. Finally, remove non-hybridized, single-strand probes thoroughly by incubating the reaction mixture with Exonuclease I (20U/μl, 2μl) at 37 °C for 30 min.

2.4.5. Color development

The above digested mixture is 50% diluted with 1.5 M NaCl. Spot a specific volume (0.5 μL) of the diluted mixture onto a nylon membrane with a micropipettor, then air dry for 10 or 5 min at 50 °C. Crosslink with ultraviolet 90 s (Energy: 3,000, CL-1000 Ultraviolet crosslinker) at room temperature. Next, block the membrane with 10% BSA in 1× TBS (pH 7.2) for 30 min at room temperature. Then, incubate the membrane with a specific volume (1 ml) of ABC–AP or ABC–HRP at 37 °C for 30 min or 1 h at room temperature. (Prepare the ABC reagent according to the manufacturer's instructions: we dilute it 1:1,000 with 5% BSA and 0.1% Triton-X-100 in 1× TBS (pH7.2)). Wash the membrane in 1× TBS buffer containing 0.1% Triton-X-100 and 0.05% Tween-20 for 3 min, six times. Finally, cover the membrane with 1 ml of BCIP/NBT, DAB, or ECL

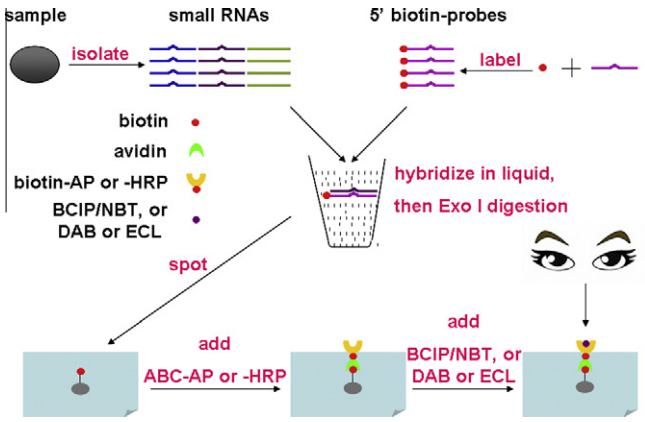

Fig. 1. Schematic diagram of procedures. Purified small RNAs are hybridized in buffer with 5′ biotin-labeled DNA probes. The mixture is spotted on a nylon membrane after digestion of non-hybridized probes with Exonuclease I. The membrane is then incubated with ABC and is developed with BCIP/NBT, DAB, or ECL to produce colorimetric end products that can be seen by eye.

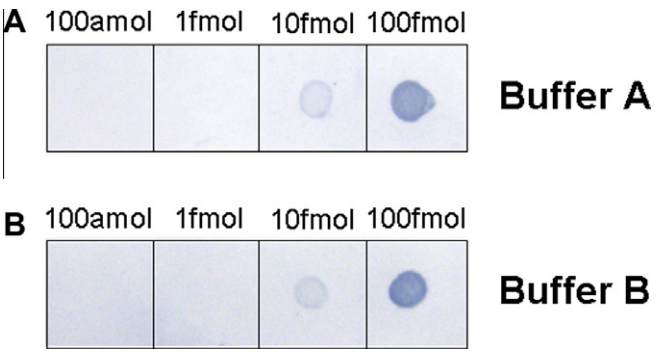

Fig. 2. Selection of hybridization buffers. (A) Buffer A is 30 mmol/L sodium phosphate buffer (pH 8.0), 0.3 mol/L NaCl, and 10 mmol/L EDTA. (B) Buffer B is 1× Exonuclease I buffer. The indicated amounts of a synthesized 22-nt small RNA (UCGGUCAGUCUGGGGCAGGCAA) and its antisense 5′ biotin-labeled DNA probe (gtTTGCCTGCCCCAGACTGACCGA) were hybridized in the indicated buffers at 55 °C, digested with Exonuclease I, and 0.5 μl of each reaction was spotted on the membrane. Alkaline phosphatase-labeled ABC was employed and BCIP/NBT was selected for color development.