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A highly sensitive and selective homogenous assay for profiling microRNA expression

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ABSTRACT

A highly sensitive and selective homogeneous microRNA (miRNA) assay is described in this report. It is based on the cleavage of DNAzyme moieties from miRNA-hybridized DNAzyme-capped capture probes (DZ-CPs) from magnetic beads by a duplex-specific nuclease (DSN). After removing the magnetic beads together with the unreacted DZ-CPs by using a permanent magnet, amplified colorimetric detection of the target miRNA is realized through the cleaved DNAzyme moieties-catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine. The exceptional amplification power of the DSN and the cumulative nature of the signal generation process permit the colorimetric detection of miRNAs down to subfemtomolar levels. The isothermal amplification scheme together with the simple assay protocol makes direct profiling miRNA in real-world samples possible.

1. Introduction

MicroRNAs (miRNAs) are ~22 nucleotides (nt) endogenous noncoding RNAs found in animals and plants (He and Hannon, 2004). The first miRNA, lin-4, was discovered by Lee et al. (1993) some 20 years ago, but many believed that was an anomaly and would not occur frequently in nature. It was not until the turn of the 21st century the regulatory role of miRNAs was confirmed before it became clear to the academics that a new research field in molecular biology has emerged (Calin et al., 2002; Reinhart et al., 2000). Growing evidence has suggested that miRNAs not only regulate protein expression, but also play an important role in many pathophysiological processes and pathogenesis of most human malignancies (He et al., 2007; Esquela-Kerscher and Slack, 2006) and specific dysregulations of certain miRNAs are seen in specific types of cancer (Lu et al., 2005). Recent studies have also revealed that miRNAs exist not only in cells but also in a variety of body fluids, including blood (Mitchell et al., 2008), saliva (Park et al., 2009), and urine (Gidlof et al., 2011). As extracellular miRNAs are easy to be detected, miRNAs are currently being evaluated as potential biomarkers in disease diagnosis, prognosis, and therapy.

Explosive activities in miRNA research have fueled the urgent demand for reliable and ultrasensitive assays for miRNA expression analysis in decentralized settings. Ideally, the miRNA assays should fulfill the following requirements: (i) sensitive enough to provide quantitative information even with small amounts of starting materials, (ii) specific enough for reproducible quantification of miRNAs down to single-base mismatch level, and (iii) easy to perform and do not require the need for expensive reagents or equipment. Unlike other nucleic acids such as DNA and messenger RNA, the unique attributes of miRNAs such as their extremely short lengths, high similarities among members of a miRNA family, and inherently different melting temperatures make it a challenging task to selectively detect minute amounts of miRNAs. As the number of miRNAs has been growing exponentially in recent years, the tedious and insensitive miRNA expression profiling techniques used in the early days of miRNA research such as molecular cloning (Takada et al., 2006) and Northern blotting (Valoczi et al., 2004) quickly gave ways to more sensitive and efficient techniques like miRNA microarrays (Thomson et al., 2004) and quantitative polymerase chain reaction (qPCR) (Chen et al., 2005; Shi and Chiang, 2005). In principle, microarray is an ideal platform for profiling miRNAs on a global scale due to its exceptional multiplexing capacity. Unfortunately, the requirements of relatively large sample size and miRNA labeling, unrealistically lengthy hybridization time, and excessive variations between protocols basically restrict the use of the miRNA microarrays in basic research in centralized laboratories (Baker, 2010). On the other hand, after extended sequences appropriate for PCR amplification are produced through several miRNA lengthening strategies (Chen et al., 2005; Shi and Chiang, 2005), the exceptional amplification power and quantitative ability of qPCR have enabled miRNAs to be profiled at single cell levels (Tang et al., 2006). Quantitative PCR is slowly becoming the method of choice for

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miRNA expression analysis. In spite of the excellent sensitivity and acceptable specificity, widespread applications of qPCR in routine miRNA expression profiling at point-of-care are severely hindered by the requirements for special laboratory skills in miRNA sample preparation and for the complex instruments in signal readout (Pritchard et al., 2012). Moreover, the accuracy of qPCR is largely dependent on proper data normalization since numerous variables inherent to a qPCR experiment need to be controlled in order to differentiate experimentally induced variations from true biological changes since certain alleles are amplified better than others under PCR conditions. Also, the thermal cycling in qPCR suggests that it only works with highly purified RNA samples as the polymerase is easily deactivated by thermally unstable co-existing materials such as proteins in pristine biological samples.

In an effort to bring miRNA expression profiling one step closer to point-of-care, an avenue that has recently been explored is the development of homogeneous miRNA assays in conjunction with isothermal amplification strategies such as Taqman probe-based fluorometry (Yin et al., 2012), single-stranded DNA binding protein-assisted capillary-electrophoresis (Dodgson et al., 2012; Wegman and Krylov, 2011), and colorimetry (Shen et al., 2013). The intrinsic poor reproducibilities and narrow dynamic ranges encountered in the heterogeneous miRNA assays can be conveniently overcome by the homogeneous assays. In addition, the isothermal amplification would effectively alleviate the above-mentioned problems in qPCR and thus substantially simplify the procedure. The colorimetric detection would significant reduce the cost and complexity of signal readout, allowing miRNA expression profiling to be routinely performed with high confidence at point-of-care. Unfortunately, most of the reported colorimetric miRNA assays are not sensitive enough to be viable alternatives. To lower the detection limit down to a level comparable to that of qPCR, here we presented a simple and robust homogeneous assay for highly sensitive and selective detection of miRNAs. A duplex-specific nuclease (DSN) was utilized to amplify miRNA hybridization events under isothermal conditions and to improve the mismatch discrimination capability of the assay. To further enhance the sensitivity, DNAzyme moieties were coupled to the DSN amplification process, thus forming a dual-amplification strategy – miRNA hybridization event amplification by the DSN and signal amplification by the DNAzyme. Compared with the thermal amplification strategies like qPCR (Pritchard et al., 2012) and ligase chain reaction (Barany, 1991), the isothermal amplifications via the DSN and DNAzyme together with its excellent mismatch discrimination ability are advantageous as they require little attention in miRNA sample preparation. The freedom from miRNA tagging and thermal cycling greatly increase the suitability in profiling miRNAs in real-world samples at point-of-care.

2. Experimental section

2.1. Materials and apparatus

Hemin (>98%), 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC, >99%), DL-dithiothreitol (DTT, >99%), and 3,3′,5,5′ tetramethylbenzidine (TMB) were from Sigma-Aldrich (St Louis, MO). Long-arm carboxyl-terminated magnetic beads (~1 μm in diameter, 20 mg/ml) were purchased from Bioclone Inc. (San Diego, USA). The DSN was purchased from Genomax Technologies Pte Ltd. (Singapore). Synthetic miRNAs, amine-terminated and DNAzyme-capped capture probes (DZ-CPs), and all other oligonucleotides of PCR purity were custom-made by Integrated DNA Technologies Inc. (Iowa, USA). A pH 8.0 50 mM Tris–HCl buffer containing 20 mM $MgCl_2$ and 1.0 mM DTT (TMD buffer) was used as the hybridization/DSN incubation medium. All other reagents of analytical grade were from Sigma-Aldrich are used without further purification. Nuclease-free water was used for all aqueous solution preparations. UV–vis absorption experiments were carried out using an Agilent Cary 60 UV–vis spectrophotometer (Agilent Technologies Pte Ltd., Singapore).

2.2. MicroRNA detection

The DZ-CP coated magnetic beads were prepared as previsously described (Chen et al., 2012). MicroRNA hybridization, DZ-CP cleavage, and DNAzyme-catalyzed colorimetric detection was performed as follows: to a 25-μL miRNA sample solution was added TMD buffer and 0.20 U DSN. The mixture was vortexed well and then incubated at 50 °C for 60 min. After the incubation, the magnetic beads were removed by a magnet. Subsequently, the reaction mixture was added to pH 3.5 5.0 mM TMB+30 mM H_2O_2 in methanol, bridefly vetaxed, and incubated for 60 min at room temperature in the dark. Finally, after adding 20 mM H_2SO_4 the absorbance was measured at 450 nm.

3. Results and discussion

3.1. Assay principle

Fig. 1 schematically illustrates the five principal steps of the proposed homogeneous miRNA assay: (1) the amine-terminated

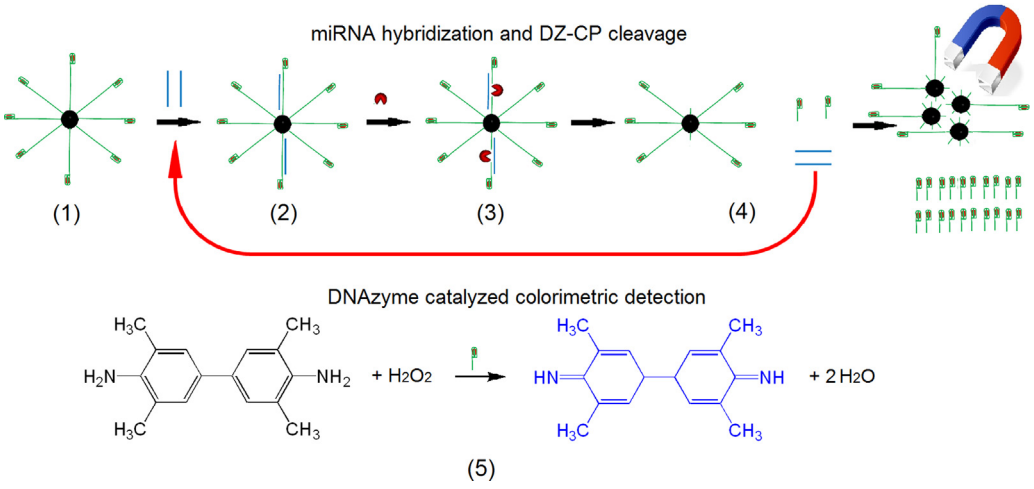

Fig. 1. Schematic illustration of the working principle of the homogeneous miRNA assay: (1) DZ-CP coated magnetic beads, (2) miRNA hybridization, (3) DSN cleavage, (4) miRNA release and recycle, and (5) colorimetric detection using TMB.