

Detection of the mature, but not precursor, RNA using a fluorescent DNA probe

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ABSTRACT

Fluorescently labelled oligonucleotide probes have been widely used in biotechnology and diagnostic field. We improved the molecular beacon probe to detect the mature, but not precursor, RNA selectively. Based on the principle that a kind of fluorophore can be quenched by adjacent guanine base, therefore, the noise fluorescence caused by the false targets can be avoided. As an example, we designed the probe for the selective detection of a mature microRNA (miRNA). We demonstrate that the probe detects mature miRNA with high specificity. Since the probe itself does not fluoresce but becomes fluorescent upon hybridization to the mature target RNA, it provides a quick and selective way for detection of various forms of RNAs.

INTRODUCTION

Methods for specific detection of nucleic acid molecules have been widely developed in basic and diagnostic biological research. Fluorescently labelled probes are used to detect single nucleotide polymorphism (SNP) and visualization of RNAs *in vivo*. The diagnostic target is recently expanded to non-coding RNA, including microRNAs (miRNAs).

MiRNAs are approximately 22-nucleotide (nt) RNAs that play important roles in regulation of fundamental cellular processes such as cell differentiation, cell proliferation and apoptosis through modulation of gene expression. The miRNA pathway begins with the transcription of a primary miRNA (pri-miRNA) by RNA polymerase II from miRNA genes. It contains a typical stem-loop structure that is processed by a nuclear enzyme complex including Drosha, and releases a generally 70-nt precursor miRNA (pre-miRNA). Once the pre-miRNA is transported to the cytoplasm, the pre-miRNA is further processed by a ribonuclease, Dicer, to yield the 22-nt mature miRNA, which is then incorporated into the RNA-induced silencing complex (RISC). RISC-bound miRNA directs the cleavage and/or the translational repression of mRNAs, thus providing a post-transcriptional control of gene expression.

Characterization of miRNAs and their expression levels in different biological samples can provide clues on their physiological functions. However, knowledge regarding the characterization and expression patterns of miRNAs is

currently restricted to a subset of known miRNAs. This is due in part to limitations of current miRNA detection assays.

The technical problems for detection of miRNAs in cellular RNAs are caused by their small size and often low abundance. The general techniques for the detection and the isolation of miRNAs from cellular RNAs are commonly used for other small RNAs. A first step could be spin-column fractionation of RNA to remove larger RNAs. After initial preparation, specific miRNAs in the sample can be detected by techniques such as Northern analysis, RT-PCR and microarrays. Northern analysis is still the standard way for the detection and the quantification of miRNA levels since the analysis clarifies the size of RNAs, and discriminates the mature RNAs from their precursors. Even though this technique is highly sensitive and quantitative, it is time consuming and, often needs radio-isotopes. Although RT-PCR has the advantage of simpler detection, but it is still ambiguous and does not discriminate between the mature and precursor miRNA. Most of the microarray approaches require upfront enrichment of small RNAs, reverse transcription, PCR amplification, fluorescent labeling and clean-up steps which are tedious and time-consuming. The other drawback of microarray

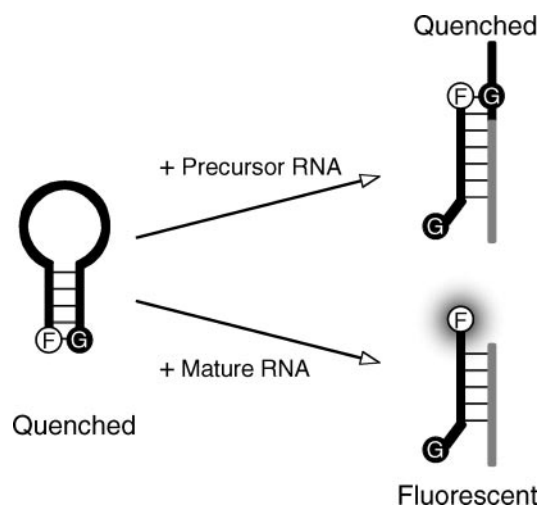


Fig. 1 Schematic representation of molecular beacon probe. One end of the probe is cytosine covalently linked with a fluorophore BODIPY[®] FL (F), which is quenched by the adjacent guanine base (G) through the base-pairing. The probe is designed to be quenched due to the guanine base in the precursor RNA. Only when the mature RNA is hybridized, the probe fluoresces.

approaches is a limitation imposed by stringent hybridization conditions when using very small molecules.

An miRNA exists in three forms, i.e., a short mature miRNA, a pre-miRNA and a pri-miRNA. To detect only the mature miRNA, an elimination of the signal from the pre-miRNAs and pri-miRNA forms is required, especially in case of the visualization of the subcellular localization. Most of the current techniques for the miRNA detection do not provide such distinction.

In this study, we have developed a novel method for detection of mature miRNAs. The probe is based on molecular beacon approach (1) using a single-stranded DNA molecule that forms a stem and loop structure. A terminus of the probe is labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY[®] FL), whose fluorescence is quenched by the adjacent guanine base(s). When the probe is hybridized with either pri-miR or pre-miR, its fluorescence was quenched by the guanine in the precursor with complementarity to the probe. Hybridization of the probe with a mature miRNAs which have no complementary guanine was resulted in fluorescent emission. Since the probe emits fluorescence only in the presence of target mature RNAs, but not in the presence of their precursors, we designated the probe as "molecular spotter" probe.

RESULTS AND DISCUSSION

The principle of molecular spotter probes is based on a molecular beacon probe using a single-stranded nucleic acid molecule that acquires a stem and a loop structure. The loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target miRNA. The stem is formed by base-pairing of two complementary sequences that are on either side of the probe sequence (Fig. 1). In the molecular spotter probe, the sequences of 3'-stem are selectively designed to complementary to the sequences of primary or precursor of miRNA but not that of mature miRNA.

The cytosine base at one end of the probe is covalently linked with BODIPY[®] FL (Molecular Probes). The fluorescent emission from the probe modified with BODIPY[®] FL was diminished after hybridization with adjacent guanine bases. We can use this phenomenon to develop method for detection of miRNA by synthesizing probe containing a BODIPY[®] FL.

To test the molecular spotter approach, we designed the molecular spotter probe for a specific miRNA, miR133. The probe possesses a complementary sequence including 3'-end labeled-cytosine base which is complementary to the guanine base in the pre-miR133 but not in the mature miR133. Since the probe designed to form a stem-loop structure, the BODIPY[®] FL becomes close to and quenched by the guanine bases via the base-pairing. The probe itself does not emit fluorescent under the condition with 100 mM NaCl and 10 mM Tris-HCl (pH 7.5) at 20°C (Fig. 2, Probe).

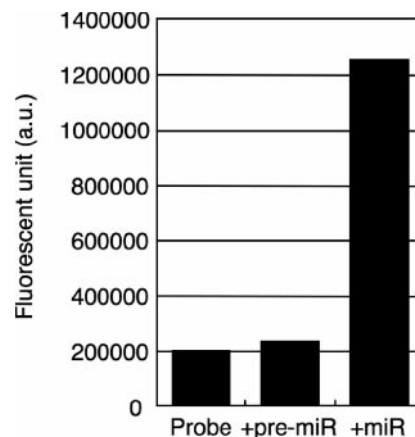


Fig. 2 Fluorescent levels of molecular spotter probe. After mixing the probe (200 nM) with oligonucleotide (400 nM) with the precursor form of miRNA (+pre-miR) or with the mature form of miRNA (+miR). Fluorescence was detected at 20°C.

Fluorescence was detected by Realtime PCR Systems 7500 (Applied Biosystems)

When the probe was hybridized with the oligo-deoxyribonucleotide whose sequence is a part of pre-miR133, its fluorescence was quenched by the guanine bases in the pre-miR133 (Fig. 2, +pre-miR). The fluorescence of the probe was observed after the hybridization with the oligo-deoxyribonucleotide whose sequence is mature miR133 (Fig. 2, +miR).

The molecular spotter probe can hybridize to both the mature and precursor miRNA sequences; the fluorophore of the probe is quenched by its adjacent guanine base. Hence, the elimination of the probe from the sample solution is not needed for the specific detection of target RNA.

CONCLUSION

The molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by self-complementary termini, and carries a fluorophore and a quencher (1). In comparison with the molecular beacon, since the natural guanine base is the quencher of BODIPY[®] FL fluorophore, the additional quencher molecules such as Dabcyl are not necessary. Direct fluorescence detection without any enzymatic reactions allows real-time quantitative monitoring of the target RNAs.

REFERENCE

1. Tyagi, S., Kramer, F.R. (1996) *Nat. Biotechnol.*, **14**, 303-308.