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Stem–loop probe with universal reporter for sensing unlabeled nucleic acids

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Abstract

In this article, we present the design principles and application of a motif composed of a stem–loop probe (SP) hybridized to a fluorescently labeled universal reporter (UR) for sensing unlabeled nucleic acids. At room temperature, SP–UR is in the hairpin-closed form in which the fluorophore of UR is in proximity to the G bases of the hairpin, where consequently the fluorescent emission is quenched significantly. On hybridization with target, SP–UR is trapped in the hairpin-opened configuration in which the fluorophore and the G quenchers are apart. This turns off quenching, increases emission intensity, and signals the presence of target. Compared with the common approach that employs an oligonucleotide probe with a covalently linked fluorophore, the use of a fluorescently labeled universal reporter strand hybridized to an unlabeled stem–loop probe provides a more efficient approach to the fabrication of nucleic acid sensors and microarrays potentially useful for real-time analysis.

Keywords: Stem–loop probes; Nucleic acid detection; Fluorescence probes; G-base quenching; Biosensor

Biosensors for DNA or RNA have important applications in forensic identification, biological weapons detection, clinical diagnostics, biomedical research, and studies in life sciences. Techniques based on molecular beacons have attracted much attention recently because of their ability to detect unlabeled nucleic acids with high specificity and potential for real-time monitoring of analytes [1–10]. One form of a molecular beacon is a single-stranded oligonucleotide with a stem–loop or hairpin structure, dual-labeled with a fluorophore and a quencher at the ends of the strand. Hybridization with target causes a conformational change to the hairpin-opened form, keeping the fluorophore apart from the quencher and resulting in an increase in emission intensity. Modifications of the molecular beacon concept include the use of a metal substrate [11,12] or guanosine of the stem–loop [13,14] as quencher. Notwithstanding the attributes of these techniques, the manufacturing cost of microarrays [15–19] of hundreds of different probes, each modified with a fluorophore, a quencher, and an attachment group [20–23] for simultaneous detection of targets, is substantial. In this article, we present the design and studies of a motif composed of a stem–loop probe hybridized to a universal reporter for sensing unlabeled oligodeoxynucleotide (Fig. 1).

The stem–loop probe (SP)[1] is a single-stranded oligonucleotide that has a hairpin structure, a reporter-binding region, and an optional address-binding region (Fig. 1A). The stem–loop region has a sequence that varies in different probes serving two functions. First, the loop and the stem

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[1] *Abbreviations used:* SP, stem–loop probe; UR, universal reporter; IDT, Integrated DNA Technologies; mRNA, messenger RNA; NHS, *N*-hydroxysuccinimide; TE buffer, Tris–EDTA buffer.

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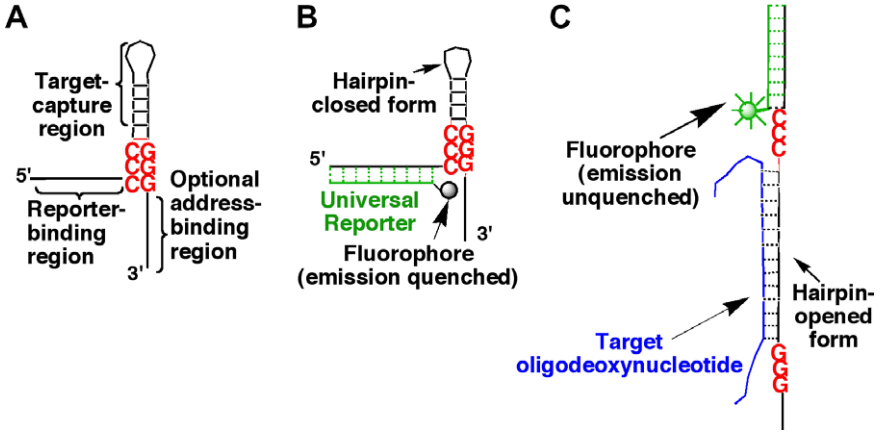

Fig. 1. Schematic representation of an SP (A), a sensing motif composed of an SP and UR hybrid (SP–UR) (B), and detection of target by SP–UR (C).

(a shared stem design [24]) or the loop region exclusively is the site for target recognition and capture. Second, the guanosines (G, labeled in red) strategically introduced at the base of the stem act as fluorescence quenchers by photoinduced electron transfer [13,14,25]. Connected next to these G quenchers is the optional address-binding region with a variable sequence for hybridization with specific address oligonucleotides if immobilization of the probe on a solid support is desired. A key component of SP is the reporter-binding region, which is a constant region with a base sequence common in all probes. The sensing motif (SP–UR) is self-assembled by hybridization of the reporter-binding region with a universal reporter (UR) strand, that is, a fluorescently labeled, single-stranded oligonucleotides (Fig. 1B). At room temperature, SP–UR is in the hairpin-closed form in which the fluorophore of UR is in proximity to the G bases of the hairpin, where consequently the fluorescent emission is quenched significantly. (For an example of the use of a universal template probe dual labeled with a fluorescent reporter on the 5′ end and a fluorescent quencher dye, instead of G bases, on the 3′ end in real-time quantitative PCR studies, see Ref. [26].) Hybridization with target traps SP–UR in the hairpin-opened form in which the fluorophore and G quenchers are apart (Fig. 1C). This turns off quenching, increases emission intensity, and signals the presence of target.

The unique design of SP–UR offers the following advantages. First, the use of unlabeled sequence-specific probes increases the yield and reduces the cost of the probe. Second, the use of a single sequence of fluorescently labeled universal reporter for all targets, instead of covalently linking the fluorophore and quencher to each probe sequence, allows efficient synthesis of the reporter in bulk quantities. Third, the self-assembly (via hybridization) of the sensor motif simplifies the manufacturing process. Fourth, the address-binding region allows immobilization by hybridization to specific address sites of microarrays without the need to modify the probe with an attachment group. Fifth, all sensing components can be affixed to a solid substrate where no washing steps are required in the detection of unlabeled targets. Sixth, the fluorescence quenching output is reversible. All of these properties minimize the time, cost, and error inherent in the analysis and also allow real-time in situ monitoring.

Materials and methods

Design and synthesis of oligonucleotides

The oligonucleotide sequences listed in Table 1 were used to illustrate the self-assembly and sensing mechanism of SP–UR. SP^{I} was designed for targets T1, T2, and T3. SP^{II} was designed for a GC-rich target sequence T4. Bases in targets that are complementary to the target-binding site of SP^{I} or SP^{II} are shown in blue. Bases in the reporter-binding, address-binding, and stem regions of SP^{I}, SP^{II}, and a control probe (CSP) are in italics, lowercase, and underlined, respectively.

The sequences of oligonucleotides were designed with the aid of OligoAnalyzer 3.0 software from Integrated DNA Technologies (IDT, Coralville, IA, USA). Sequences were designed to achieve specific melting temperatures and to minimize the formation of unwanted hairpins, cross-hybridization, and self-dimers. Caution should be taken in the design of the SP to avoid GC-rich sequences and sequences that may form unusual structures such as telomeric repeats.

The sequences of UR and the reporter-binding site are designed to ensure that UR remains hybridized to SP^{I} or SP^{II} under normal operating conditions and to minimize fluorescence quenching when SP^{I}–UR and SP^{II}–UR are in the hairpin-opened form. The target-binding site of SP^{I} was designed for detecting the murine B7.2 messenger RNA (mRNA) sequence (NCBI L25606), and the target-binding site of SP^{II} was designed for detecting the CDKN1A (p21) mRNA sequence.

The oligonucleotides were custom synthesized by IDT. TAMRA-labeled UR was custom synthesized by IDT by coupling a TAMRA *N*-hydroxysuccinimide (NHS) ester to the 5′ end of the oligonucleotide sequence of UR that