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Biofunction-assisted DNA detection through RNase H-enhanced 3′ processing of a premature tRNA probe in a wheat germ extract

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ABSTRACT

We have developed a novel type of biofunction-assisted, signal-turn-on sensor for simply and homogenously detecting DNA. This sensor system is composed of two types of in vitro-transcribed label-free RNAs (a 3′ premature amber suppressor tRNA probe and an amber-mutated mRNA encoding a reporter protein), RNase H, and a wheat germ extract (WGE). A target DNA induces the 3′ end maturation of the tRNA probe, which is enhanced by RNase H and leads to the expression of a full-length reporter protein through amber suppression in WGE, while there is almost no expression without the target due to the inactivity of the premature probe. Therefore, the target can be readily detected with the activity of the translated reporter. The catalytic reuse of the target with the help of RNase H in addition to various bioprocesses in WGE enables this sensor system to exhibit relatively high selectivity and sensitivity.

In conjunction with the increase in the number of identified genetic markers, numerous gene-sensing methods have been developed for detecting these markers. Recently, attention has been focused on simple and homogeneous methods that do not utilize nucleic-acid amplification such as polymerase chain reactions (PCRs).[1] Molecular beacon (MB)-based methods are representative of this approach.[2] Although a classical MB probe that is composed of end-labeled hairpin DNA exhibits a relatively high detection limit due to the one-to-one stoichiometry, some inventive strategies have been developed to improve the sensitivity.[3–5] For example, cycling probe technology (CPT) enables a target DNA to catalytically cleave an MB-like DNA–RNA–DNA chimeric probe with RNase H, which leads to a higher sensitivity.[3] CPT also shows a high selectivity suitable for single nucleotide polymorphism (SNP) detection, because RNase H cleaves only RNAs in full-matched DNA/RNA duplexes.[3c] Another strategy for signal amplification is the utilization of catalytic bioprocesses, specifically ribosomal translation and the subsequent enzymatic reaction of a reporter protein.[5–8] Sando et al. pioneered a biofunction-assisted sensor based on this strategy by harnessing a reconstituted prokaryotic cell-free translation system.[5a] In this sensor, the signal is amplified through the two continuous catalytic bioprocesses that are induced by target-dependently opening an MB-like but label-free hairpin-mRNA probe, which encodes a reporter protein. We recently constructed an advanced, biofunction-assisted sensor by combining CPT with eukaryotic cell-free translation in wheat germ extract (WGE).[8,9] This advanced sensor employs four catalytic processes including RNase H-mediated cleavages of the chimeric probe and mRNA, and thus shows much higher sensitivity. However, the chimeric probe is very expensive, and, what is worse, the signal-turn-off mechanism through mRNA digestion involves a higher risk of false positives, because non-target materials tend to inhibit biofunctions. We report herein a novel type of biofunction-assisted 'signal-turn-on' sensing method, in which a readily-preparable, in vitro-transcribed tRNA precursor (i.e., premature tRNA or pre-tRNA) is used as a probe in combination with RNase H and WGE.[9]

As the framework for our tRNA probes, we chose an amber suppressor tRNA named **t86** (or S2-$G_{27}C_{43}$-G_{73}), which was derived from *Oryza sativa* nuclear $tRNA^{Ser}$ (Supplementary Fig. S1A).[10] **t86** is aminoacylated with Ser by endogenous wheat seryl-tRNA synthetase and suppresses the amber codon (UAG) on mRNA with a high efficiency (60–85%) in WGE. Therefore, its presence or production can be readily detected with the activity of protein that is translated from mRNA with one amber codon in the open reading frame (amber-mRNA).[11] In fact, we previously applied this assay to **t86**-based pre-tRNAs to investigate their end processing in WGE.[12] The results clearly showed that 3′ processing (i.e., the RNase Z-mediated 3′ trailer cleavage and the $C_{74}CA$ addition) took place rapidly.[13] Specifically, a **t86**-based pre-tRNA with an original linear 7-nt 3′ trailer (U_{74}UUGCUU) at its 3′ end (**3pt**, Supplementary Fig. S1B) exhibited a suppression efficiency as high as that by **t86**, despite the fact that **3pt** should not exert its function as a

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suppressor without undergoing the 3′ processing. In contrast, when an extra inhibitory sequence (IS) was added to the 3′ end of **3pt** so that it sequestered the cleavage site (G_{73}-U_{74}) by forming a stem-loop (IS-SL) with the 3′ region, the suppression efficiency was decreased to 10% of that by **3pt**, depending on the IS-stem length (up to 14 bp) (**3pt-IS(14)**, Fig. 1A).[7] This means that the 3′ processing (and the subsequent amber suppression by the resulting mature **t86**) can be regulated by modulating IS-SL formation.[14]

Based on the findings described above, we rationally designed an MB-like tRNA probe for DNA detection (**3pt-X(16)**: X = target DNA) by altering the 3′ trailer segment of **3pt-IS(14)** (Fig. 1A). Concretely speaking, the loop of IS-SL and the following 6 nt of the stem (red Ns) were assigned to the target DNA-binding site to target-dependently distort the IS-SL. Although it is necessary to also change the original 3′ trailer part (blue Ns) for IS-SL formation without the target, the 3′ processing efficiency is almost independent from the sequence of this part, except for the 74th base (Supplementary Fig. S2).[15] In addition, to ensure the target binding, the loop was lengthened to 15 nt, in view of the fact that 14–16 nt are required for complete RNA-DNA hybridization even at μM concentrations in WGE.[8] In this regard, the IS-stem was extended by 2 bp to compensate for the destabilization of IS-SL caused by the loop extension.[16] We then paired this tRNA probe with an amber-mRNA encoding the firefly luciferase (Fluc) gene (**amber-mRNA(Fluc)**) in WGE to develop a novel-type biofunction-assisted biosensor for DNA detection (Fig. 1B). In this sensor, a target DNA is expected to bind to the tRNA probe to open the IS-SL, which should induce the 3′ processing of the probe and subsequently turn the Fluc expression 'on' through amber suppression. Moreover, it is highly likely that RNase H helps a target to be catalytically used for the IS-SL cleavage and enhance the output signal, as in CPT (the dashed arrow in Fig. 1B).[3,8]

In order to precisely compare the performance of the present sensor system to those of some previously reported biofunction-assisted sensors, we prepared a tRNA probe (**3pt-CCR5(16)**, Fig. 2A) for detecting a region around the 627th nucleotide of the HIV-related human chemokine receptor 5 (CCR5) gene (Fig. 2B),[17] which was targeted by the previous sensors.[5,8] The target amount was initially set at 100 fmol, which is 500-fold less than that of the tRNA probe. After the probe was pre-incubated with (or without) the target in the presence (or absence) of 8 U of RNase H for

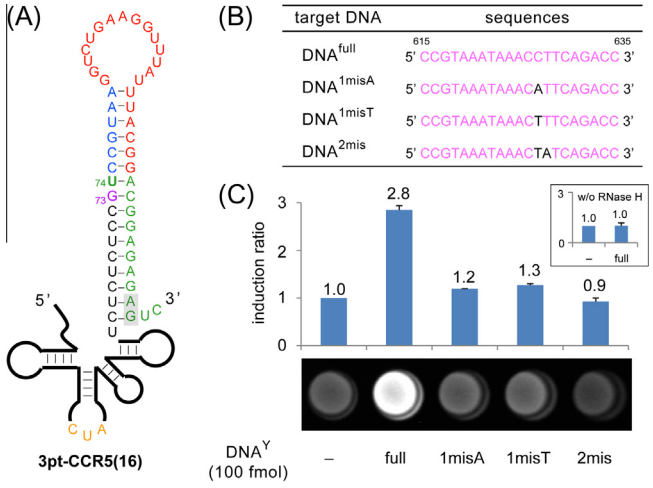

Figure 2. Detection of the 615–635 region of the CCR5 gene. (A) Schematic diagram of the **3pt-CCR5(16)** probe, which was designed based on the strategy in Figure 1A. (B) The sequences of the target DNA and its several mutants. (C) Induction ratios of the Fluc expression (i.e., the amber suppression) in the presence of each DNA (100 fmol) and the probe (50 pmol) with (or without (inset)) RNase H treatment, relative to that in the absence of any target DNA (leftmost). Representative chemiluminescence images of the samples are shown below the graph.

1 h,[18] WGE and **amber-mRNA(Fluc)** were added to the solution and the mixture was further incubated for 1 h for cell-free translation. The expression efficiency of Fluc was then measured with luciferin to evaluate the target-dependent amber suppression. The results showed that the full-matched target (DNA^{full}) enhanced the suppression efficiency approx. 3-fold compared to that in its absence, whereas it could not induce the amber suppression without the use of RNase H (Fig. 2C). This indicates that such a small amount of target can be well detected by being catalytically reused with the help of RNase H. It should be noted that the same amount of DNA^{full} could also be detected in the co-presence of 100-fold larger amounts of non-target DNA with RNase H, though the induction ratio decreased slightly (Supplementary Fig. S3). In contrast, three (two 1-nt and one 2-nt) mismatched targets (DNA^{1misA}, DNA^{1misT} and DNA^{2mis}, Fig. 2B) had almost no effect on the

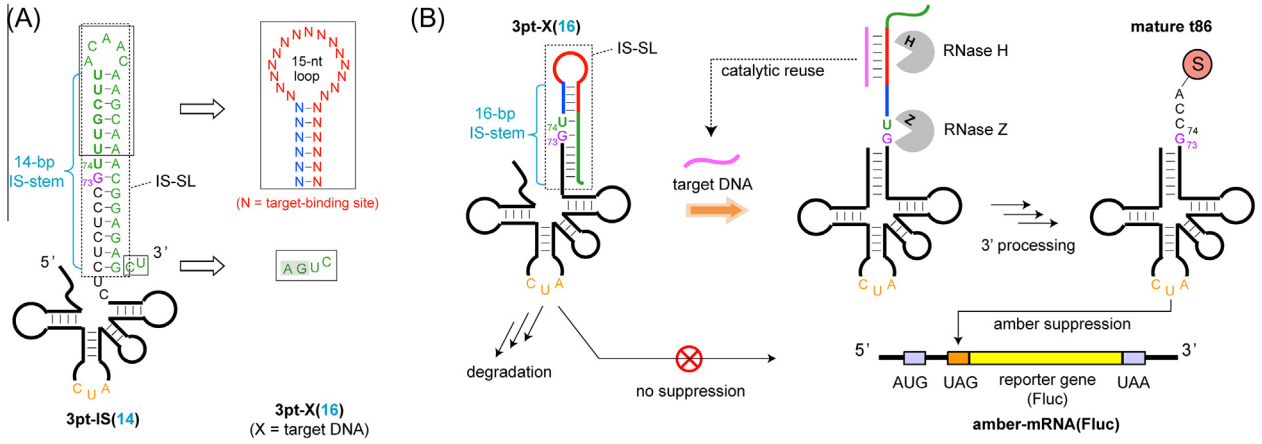

Figure 1. (A) Design of a tRNA probe for DNA detection (**3pt-X(16)**, X = target DNA) based on **3pt-IS(14)**,[7] which is not susceptible to the 3′ processing in WGE due to the stable IS-stem. The dashed box and solid boxes represent the IS-SL and the altered bases, respectively. The two shaded bases (AG) in the lower right box were inserted to extend the IS-stem by 2 bp in consideration of the IS-stem destabilization by the loop extension.[16] A dinucleotide UC was added to the 3′ terminus just for precise PCR-amplification of the probe template. (B) Schematic illustration of the novel-type biofunction-assisted 'signal-turn-on' sensor in this study. While the tRNA probe mainly undergoes degradation without the target DNA in WGE (left),[12] the target binding enables the probe to restore the tRNA structure (middle) and then to be processed via the endogenous RNase Z-mediated G_{73}-U_{74} cleavage to the stable mature amber suppressor **t86** (right). RNase H catalytically releases the target from the probe by cleaving the target-binding site (dashed arrow): it enhances the target-dependent 3′ processing. Because mature **t86** efficiently suppresses the amber codon on **amber-mRNA(Fluc)** in WGE,[10] the target can be readily detected with the Fluc activity.