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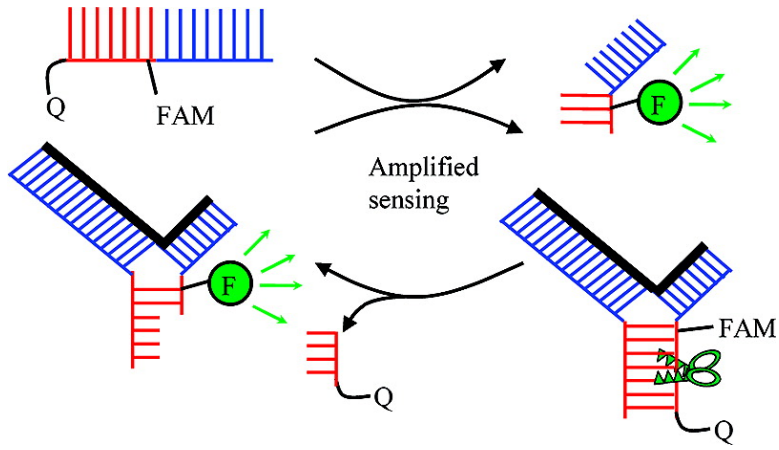

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Junction Probes − Sequence Specific Detection of Nucleic Acids via Template Enhanced Hybridization Processes

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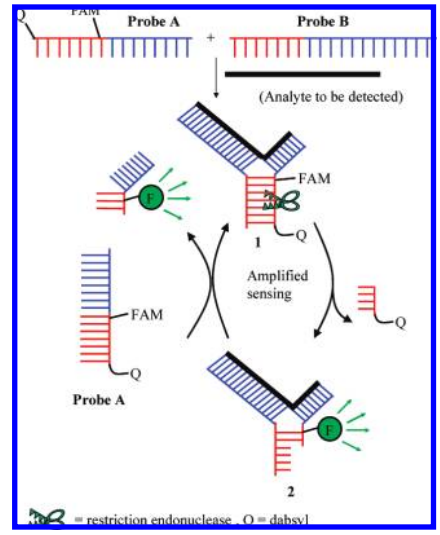
Several studies have shown that the individual differences in drug metabolism or susceptibility to diseases such as cancer and some genetic diseases are caused by single nucleotide polymorphisms (SNPs) and short insertion/deletion variations (InDels).[1] These studies have led to an increasing demand to detect specific genes in clinical samples for diagnostic purposes. The majority of commercial DNA detection methods[2] require polymerase chain reaction (PCR)[3] or ligase chain reaction (LCR)[4] steps to preamplify the genes of interest, which are usually minor components in a complex mixture of other nucleic acid sequences. However, PCR or LCR based methods have some limitations. For example, certain alleles amplify better than others under PCR conditions leading to over- or underestimation of the concentration of a target DNA sequence in a sample.[5] The use of thermal cycling in both PCR and LCR limits the adaptation of these methods for in vivo applications.

The aforementioned limitations of PCR and LCR based detection methods have spurred the development of alternative detection platforms that either do not require preamplification of the gene of interest or achieve non-PCR isothermal amplified sensing.[6−10] The majority of methods that do not preamplify the DNA target are based on the difference between the melting temperatures of perfectly matched and mismatched DNA templates.[11,12] This usually requires stringent temperature control, sophisticated instrumentation/reagents, or the need for a skilled technician. Therefore the adaptation of these technologies by other laboratories has been limited.

We herein report a proof-of-concept for a new nucleic acid detection technology called junction probes (JPs) which achieve non-PCR based isothermal amplified sensing without the need for a thermal cycling procedure. Junction probes operate *via* a concept called Template Enhanced Hybridization Processes (TeHyP), *vide infra*. TeHyP encompasses a design strategy whereby two probes that do not hybridize to each other at a specific temperature can be made to anneal to each other in the presence of a template *via* the formation of a ternary complex (Scheme 1). The resulting structure that forms after the template-enhanced hybridization can then be detected *via* a variety of means/processes (see Scheme 1 for an example).

For our first-generation JP technology, one of the detection probes, **A**, is labeled with a fluorescent molecule (FAM) that is quenched by Dabsyl. The FAM and Dabsyl molecules are separated by a 7-mer sequence (5′-TTTGATC-3′; colored red in Scheme 1) that contains a BfuCI[13] restriction endonuclease (REN) site. REN enzymes only cleave double-stranded DNA; therefore probe **A** can not be cleaved by BfuCI unless it hybridizes with a complementary strand to form a duplex. Probe **A** contains two regions (colored blue and red in Scheme 1) that are complementary to part of a DNA/RNA target (colored blue) and probe **B** (colored red). Probe **B** is unlabeled and also contains two regions: a 7-mer region (colored red in Scheme 1) that is complementary to part of probe

Scheme 1. Amplified Sensing Using TeHyP Concept



A and a region that is complementary to part of a DNA/RNA target (colored blue in Scheme 1). The melting temperature of the duplex that results from probe **A** annealing to probe **B** is lower than 15 °C (see Supporting Information for thermal melting curves). Therefore, it was expected that in the absence of a template that can enhance the hybridization of probes **A** and **B**, BfuCI will be unable to cleave probes **A** and **B** because the two oligonucleotides will predominately exist as single-stranded at the reaction temperature (30 °C).[14] However, in the presence of a DNA template, probes **A** and **B** and the template will hybridize to form a ternary "Y" junction structure, complex **1** (see Scheme 1). One arm of the Y junction structure contains a cleavage site for BfuCI and will be subsequently cleaved by this enzyme. The cleavage will then result in another ternary structure **2** which has a lower stability than complex **1** (see Supporting Information for the thermal melting curve of complex **2**).

It is important to keep the region of sequence complementarity between the two detection probes to a minimum in order to avoid a template independent hybridization and a subsequent REN cleavage that will lead to high background noise. For the restriction endonuclease enzyme, BfuCI, at least one base pair is required between the REN recognition site and the junction in complex **1** (see Supporting Information). However, when the fluorescein molecule was placed on the thymine nucleotide that lies next to the recognition site (**JP3**, Figure 1) or two nucleotides away from the REN recognition site (**JP2**), no cleavage product was obtained. **JP1**, however, could be cleaved by BfuCI. We speculate that when FAM was closer to the REN cleavage site, the aromatic moiety of FAM either intercalated into the REN recognition site and retarded cleavage or provided a steric block to the enzyme latching onto the recognition sequence. Blunt ends were also not tolerated

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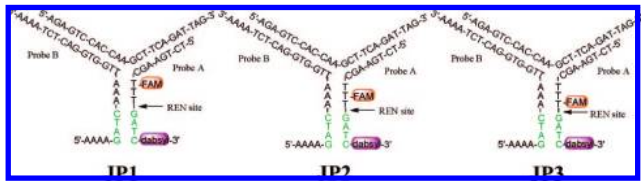

Figure 1. Structural requirements for efficient cleavage by BfuCI.

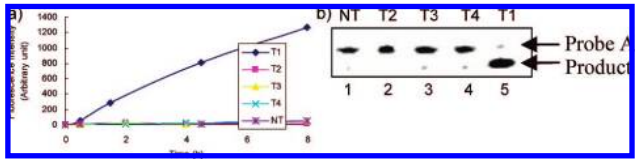

Figure 2. (a) Fluorescence based SNP detection (λ_{ex} = 494 nm, λ_{em} = 518 nm). (b) Gel based SNP detection. Reaction conditions: probes 1 μM, template 1 μM, BfuCI 0.0267 U/μL; buffer: 20 mM Tris-acetate, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, bovine serum albumin (100 μg/mL); 30 °C.[14] NT = no template was added.

Table 1. DNA Template Sequences

T1	5′-AGAGTCCACCAA GCTT**C**AGATTAG
T2	5′-AGAGTCCACCAA GCTT**G**AGATTAG
T3	5′-AGAGTCCACCAA GCTT**A**AGATTAG
T4	5′-AGAGTCCACCAA GCTT**T**AGATTAG
A	5′-TCTGAAGCT(FAM)TTGATC-Dabsyl
B	5′-AAAAGATCAAATTGGTGGACTCTAAAA

immediately after the REN site. A minimum of one base pair or overhang was needed for effective cleavage by this enzyme (see Supporting Information). Overhangs of two or more nucleotides gave a faster cleavage reaction than a single overhang (see Supporting Information).

The rate of cleavage at the REN site in the duplex region formed between probes **A** and **B** in the presence of 1 equiv of target sequence, T1, is 60 times higher than that in the absence of T1 (see Figure 2a).[15] Template detection by junction probes is also sequence selective. When single mismatch targets (T2−T4, mismatch site colored blue in Table 1) were used, the yields of cleavage products were similar to that of a target-free reaction, and only a matched template provided a significant product band on a PAGE gel (see Figure 2b). Similarly, the fluorescence intensities obtained with single mismatch targets were similar to that of the target-free reaction (see Figure 2a).

In order to test if indeed the DNA template can act as a catalyst and turn over the cleavage reaction, various template concentrations ranging from 0 to 2 μM were studied. When 0.25 equiv of matched template **T1** (500 nM) was used, the yield of the reaction was 400% (corresponding to a turnover (TO) of 4; see Supporting Information, Figure *S*6). 0.05 and 0.01 equiv of **T1** (100 and 20 nM) furnished product yields of 1323% (TO 13) and 3636% (TO 36), respectively. From these results we concluded that the template is able to catalyze the reaction under isothermal conditions.

In an alternative probe design, **JP4**, the base pairing between probes **A** and **B** was reduced to six *via* the introduction of a mismatch base pair (see Figure 3). For this approach, there was a significant change in the fluorescence intensity in the presence of target template T1 but not in the absence of T1 (Figure 3).

For our first-generation JP technology, **JP1**, a signal-to-noise ratio of 60 is comparable[6,7] or superior[8] to existing technologies.

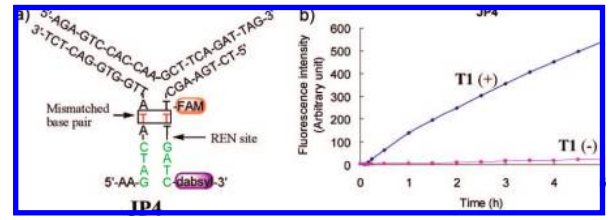

Figure 3. Reaction conditions: same as those for Figure 2.

Because of the high signal-to-noise ratio of our technology, picomolar concentrations (femtomoles) of DNA could be detected (see Supporting Information, Figure S10).

We have used the TeHyP concept to develop junction probes that can amplify DNA detection signals under isothermal conditions. Because TeHyP technology adds a second dimension to detection probes and separates the region that hybridizes to the target from the fluorogenic processing region, it has been possible to use other cheap DNA processing enzymes that are ordinarily not utilized in DNA detection.[16] In principle the TeHyP technology can be used to detect other molecules apart from nucleic acids. The only requirement is for the molecule of interest to be able to bind to a segment of the TeHyP probes.

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Supporting Information Available: PAGE gels, fluorescence data, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Commercially available from New England Biolabs Inc. See http://www.neb.com/nebecomm/default.asp. See Suppporting Information for explanation for the choice of BfuCI.
(14) The optimal temperature for BfuCI is 37 °C, but the reaction was performed at 30 °C because complex **1** is not stable at 37 °C; see Supporting Information.
(15) In order to acheive a high signal-to-noise ratio, it is important to use double distilled and DNAse-free water and also ensure that the reaction temperature does not fall below 30 °C.
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