

ABSTRACT

Title of Document: JUNCTION PROBES - SEQUENCE SPECIFIC
DETECTION OF NUCLEIC ACIDS VIA
TEMPLATE ENHANCED HYBRIDIZATION
PROCESSES

Lei Yan, Ph.D., 2011

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As new disease biomarkers such as cancer-linked microRNAs are discovered, the need for new strategies for the detection of these disease biomarkers at isothermal conditions will increase. The junction probe (JP) technology is a restriction endonuclease-based nucleic acids detection platform that achieves isothermal amplified sensing and does not require the presence of an endonuclease recognition sequence in the target analyte. The first generation junction probe platform however suffered from long assay time (several hours). We hypothesized that the slow catalysis in the first-generation JP platform was due to an inhibition cycle. Consequently, a second generation JP platform, which is modified with phosphorothioate moieties in order to suppress the inhibition cycle, was developed. The second-generation JP platform is significantly superior to the first-generation platform and we demonstrated the potential of second-generation JP by detecting

microRNA and bacterial ribosomal RNA. Importantly, second-generation JP could detect RNA in crude bacterial cell lysates without extensive sample preparation.

JUNCTION PROBES - SEQUENCE SPECIFIC DETECTION OF NUCLEIC
ACIDS VIA TEMPLATE ENHANCED HYBRIDIZATION PROCESSES

By

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Abbreviations

BSA: bovine serum albumin

DTT: dithiothreitol

FAM: fluorescein

FEN: flap endonucleases

FISH: fluorescent *in situ* hybridization

FRET: fluorescent resonance energy transfer

ISH: *in situ* hybridization

JP: junction probe

LCR: ligase chain reaction

MAPT: microtubule associated protein tau

MB: molecular beacon

PCR: polymerase chain reaction

PNA: peptide nucleic acid

QUAL: quenched autoligation

RCA: rolling circle amplification

REase: restriction endonuclease

SNP: single nucleotide polymorphism

SSB: Single-stranded DNA binding protein

TDI: template-directed dye-terminator

TeHyp: template enhanced hybridization process

TO: turn over

Chapter 1: Introduction

1.1 Importance of nucleic acids detection

Sensing nucleic acid sequences is vital in modern biology and medicine and the field has grown exponentially since the 1990s, and is likely to continue to increase in importance over the next few decades. The detection of pathogenic bacteria/viruses and cancer genes in a patient's sample can be critical to decisions that impact recovery. In this past decade, seminal discoveries made by several groups have linked the susceptibility to diseases such as cancer or the differences in drug metabolism to single nucleotide polymorphisms (SNPs) and short insertion/deletion variations.¹ Several studies have also shown that RNA plays a key role in cancer development.¹⁻⁹ Human miRNA genes are often found near cancer-associated genomic regions and fragile sites and some miRNAs have lower levels of expression in cancer samples compared to normal tissues.² Abnormal expression of miRNAs has been found in cancers including chronic lymphocytic leukaemia,³ colorectal neoplasia,⁴ paediatric Burkitt lymphoma,⁵ lung cancer,⁶ large cell lymphoma,⁷ glioblastoma,⁸ and B cell lymphomas⁹. Profiling miRNA tools are therefore required for the understanding of the expression pattern of miRNA. The detection of nucleic acid sequences without prior purification and amplification, in cells or in biological fluids, and even directly in living organisms is highly desirable. The demand to detect specific nucleic acid sequences of different lengths to aid clinical diagnosis and for understanding fundamental biology has spurred the development of myriads of DNA/RNA detection technologies.¹⁰

One of the most specific molecular recognition events, which is the basic principle for nucleic acids detection, takes place when a strand of nucleic acid anneals to its complementary partner, for example, 5'TAC anneals to 3'ATG. A single-stranded oligonucleotide probe can find a complementary strand in the presence of a large excess of other nucleic acid sequences and this process is now being applied to the field of oligonucleotide sensing.

1.2 Thermal cycling assisted detection

1.2.1 Polymerase chain reaction (PCR)

PCR, developed in the mid 1980s, is widely used for enzymatic amplification of nucleic acid sequences.¹¹ The PCR procedure is described in Figure 1.1. To a solution containing the DNA target, which could be either single or double strands, two primers that are complementary to the ends of the target sequence and deoxyribonucleotide triphosphates are added. The solution is then heated to denature the original strands and cooled down to facilitate the annealing between primers and targets. A thermostable polymerase, which is also added to the mixture extend the primers using the original strands as templates to synthesize new strands. This is the end of the first cycle. As the denaturing, annealing and polymerase extension continue, the newly synthesized strands can be utilized as templates and more copies are produced.

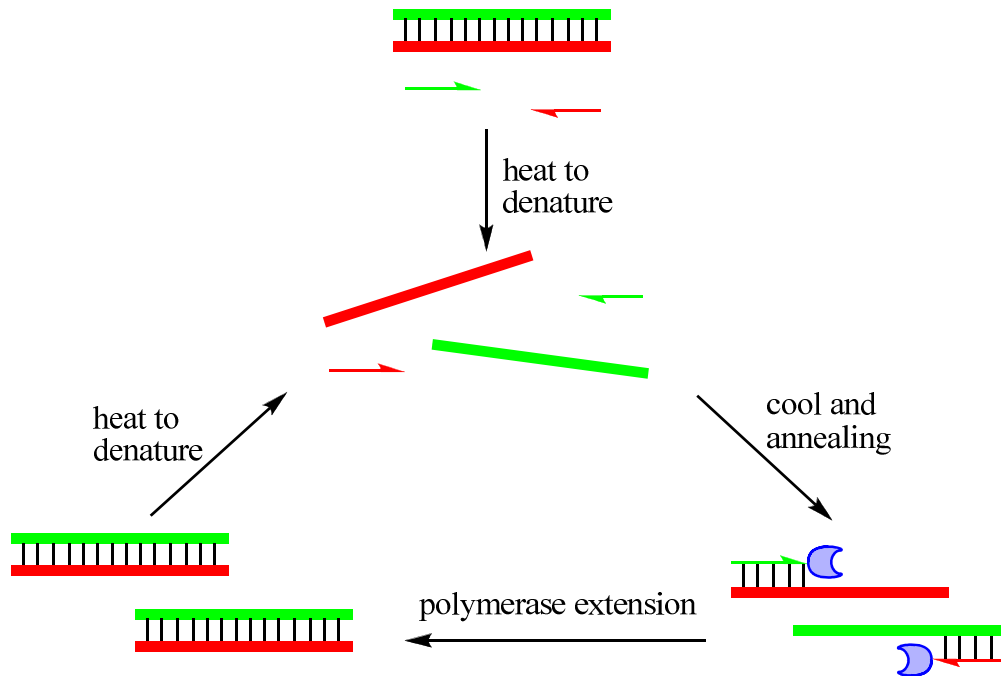


Figure 1.1 Polymerase chain reaction

Conceptually PCR provides a simple means to detect DNA. If the target is present, it should be possible to get amplified and the PCR products can be detected via a variety of ways. RNA target can also be detected by PCR after reverse transcription (Figure 1.2).¹²



Figure 1.2 RNA detection by PCR

1.2.2 Taqman probes

Several detection technologies that incorporate “fluorogenic” processes with polymerase chain reaction have been developed. A fluorogenic process is defined as a process that leads to enhance fluorescence of a non-fluorescent entity. Among the

earliest examples of PCR-based DNA detection assays was the Taqman assay, which uses Taq DNA polymerase.¹³ For the Taqman, two primers are utilized in the assay. One of the primers is used for extension whereas the other primer, which lies a few nucleotides away from the first primer when these two primers bind to the template, is labeled at one end with a fluorophore and at the other end with a fluorescence quencher (Figure 1.3). The Taq DNA polymerase has an exonuclease activity so when it reaches the second fluorophore/quencher-labeled probe, the enzyme degrades this probe, leading to the physical separation of the fluorophore and the quencher and a subsequent enhancement of fluorescence. The Taqman probe was used for identifying the pathogenic strains of *L. interrogans*.¹⁴

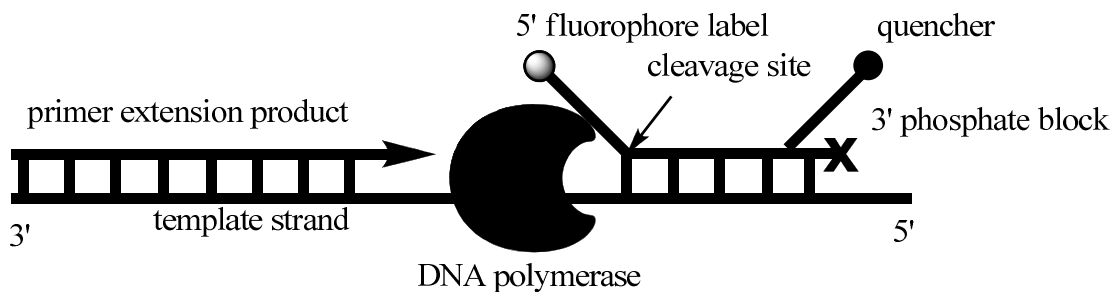


Figure 1.3 Taqman probe

1.2.3 Hybridization probes

Another direct way to monitor PCR amplification is hybridization probe assay.¹⁵ The probes are designed to be complementary to part of the PCR product and bear a pair of fluorophores which are fluorescence resonance energy transfer (FRET) partners. FRET is the process of energy transfer from an excited donor fluorophore to a suitable acceptor fluorophore. The efficiency of energy transfer depends on the extent of spectral overlap and proper dipole alignment of the two fluorophores. In the hybridization probe assay, the two probes are not close to each other and energy

transfer between the two fluorophores is less probable before their hybridization to the target nucleic acid. In the presence of PCR product, the two probes are then brought close to each other and the acceptor dye can transfer fluorescence energy to excite the donor dye (Figure 1.4).

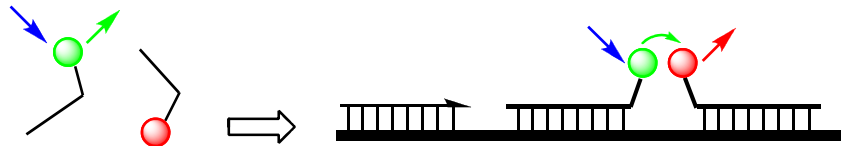


Figure 1.4 Hybridization probe

1.2.4 Scorpion probes

Scorpion probe is based on stem-loop structure and can be used to detect PCR products in homogeneous solution (Figure 1.5).¹⁶ A Scorpion probe consists of a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences on the 5' and 3' ends of the probe. The fluorophore attached to the 5'-end is quenched by a moiety joined to the 3'-end of the loop. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. It is essentially an amplifiable molecular beacon assay, *vide infra*.

Other PCR based strategies which are not covered here include Light-up probes,¹⁷ Displacement probes,¹⁸ and Light Upon eXtension primers,¹⁹ etc. The PCR based platforms have facilitated the detection of few copies of target genes in patients' samples. However, these technologies have certain limitations. For example, PCR can be allelic-biased. In particular, GC-rich regions in DNA, especially those which can

potentially form stable stem loop structure, are difficult to amplify.²⁰ Sample preparation can also be a challenge since some PCR inhibition factors are not easy to eliminate.²¹

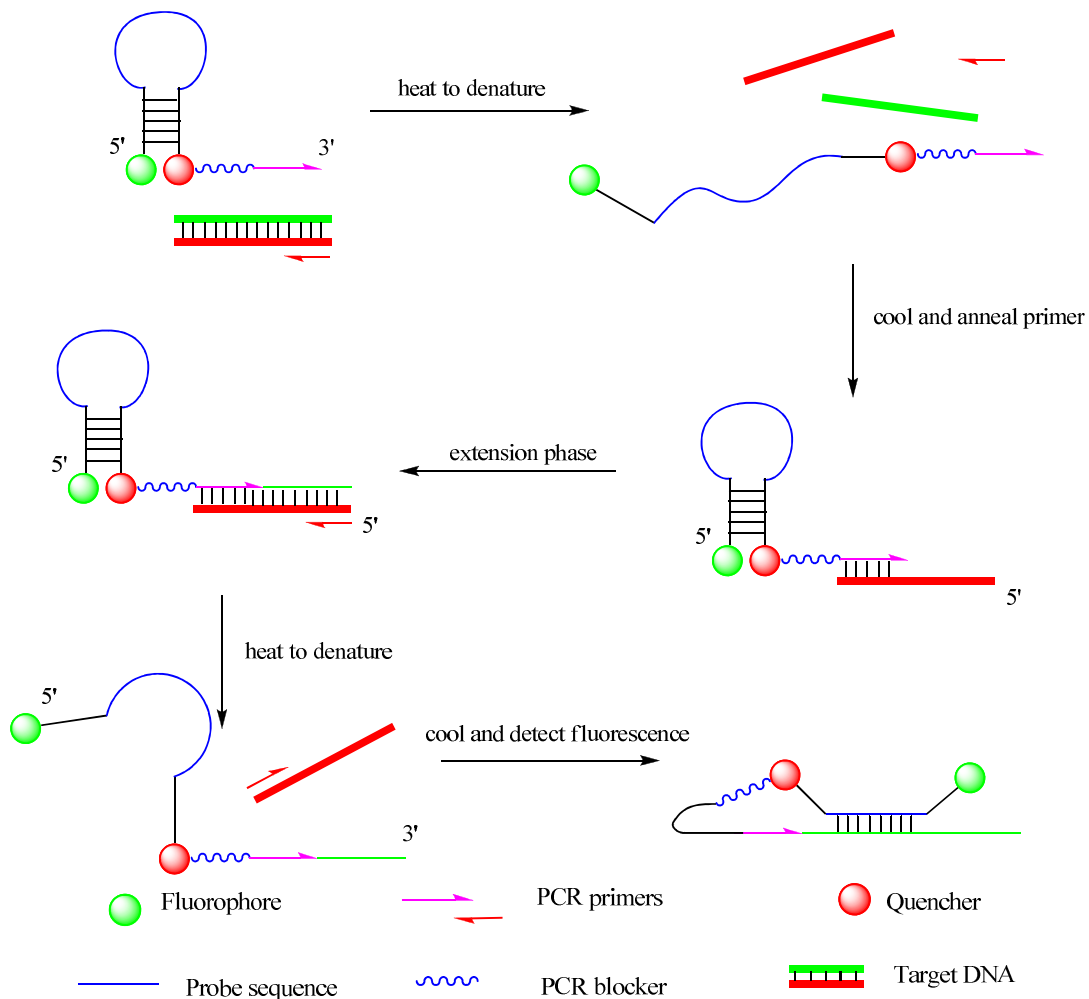


Figure 1.5 Scorpion probing mechanism

1.2.5 Ligase chain reaction (LCR)

DNA detection using two probes that can be ligated together using a ligase enzyme is also popular.²² The ligase enzyme can ligate two oligonucleotides together when the two probes anneal to the DNA target in a juxtapose manner. It is only when the

probes and the template are correctly base paired at the joining site that the ligase enzyme joins the two probes. This feature of ligase is utilized to detect SNPs. A SNP is a small genetic change, or variation, that can occur within a person's DNA sequence. The two probes can be labeled with biotin and ^{32}P respectively for separation and sensing purpose (Figure 1.6a).²² The obvious limitation of an isothermal ligase detection is the lack of amplification. Barany expanded the capability of ligase detection of DNA by developing a ligase chain reaction (LCR) using a thermalstable ligase, which allowed for an amplification cycle.²³ Like PCR, newly formed ligated products serve as new templates for unreacted probes in the amplification cycles, and mismatched target can not promote any ligation. Instead of two primers, LCR require four primers (Figure 1.6b).²³ LCR was applied as a screening tool to detect culture positive tuberculosis,²⁴ and recently conformationally modified LCR primers were designed to detect microRNA.²⁵

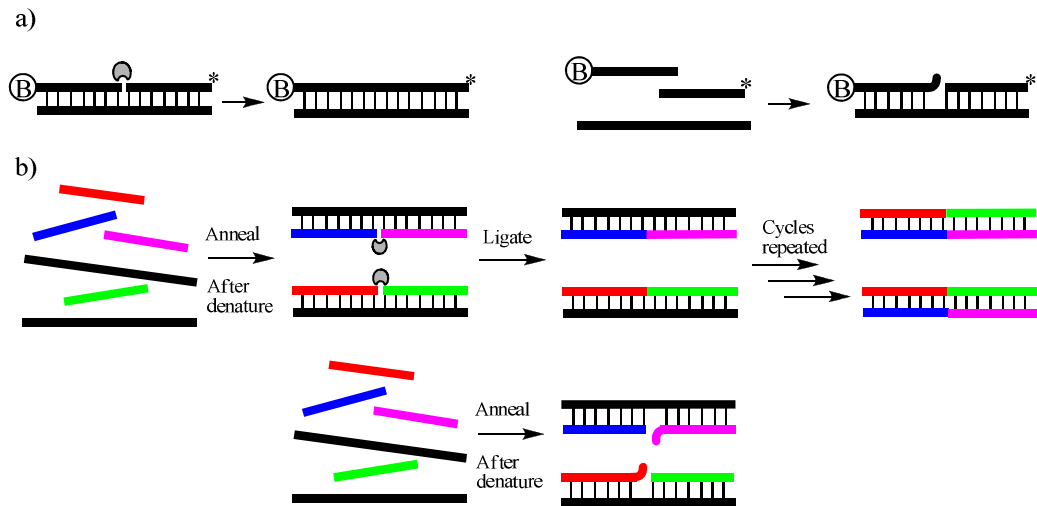


Figure 1.6 Ligase detection and chain reaction

The thermal cycling based methods, PCR and LCR, have a wide range of application because of their powerful amplification ability. However, the use of

heating and cooling processes limit the adaption of these platforms *in vivo* and in resource-poor regions whereby a thermocycler might be cost-prohibitive. Thus many groups have devoted their efforts to develop isothermal nucleic acids detection strategies.

1.3 Isothermal nucleic acids detection without amplification

1.3.1 *In situ* hybridization (ISH)

Before PCR was introduced to biology and medicine, *in situ* hybridization (ISH) was developed in early 1969 by Pardue and Gall,²⁶ in which radiolabeled DNA was read out by microautoradiography. It was introduced into bacteriology by Giovannoni,²⁷ who was the first to use radioactively labeled oligonucleotide probes for the microscopic detection of bacteria. Without changing the cell's morphology or the integrity of its various compartments, this historical technique allowed nucleic acid sequences to be examined inside cells. Radioactive labels were steadily replaced by non-isotopic dyes with the development of fluorescent labels²⁸ since better safety and resolution are provided compared to the radioactive probes. Moreover, fluorescent probes can be labeled with dyes of different emission wavelength thus enabling detection of several target sequences within a single hybridization step. In 1989, fluorescently labeled oligonucleotides were used for the detection of single microbial cells by DeLong.²⁹ Over the last decade, fluorescence *in situ* hybridization (FISH) has become a popular technology due to its improving sensitivity and rapidity for diagnostic, environmental, ecologic, and phylogenetic analysis in microbiology.³⁰

FISH detects nucleic acids sequences within the intact cell through direct specific hybridization of a fluorophore-labeled probe to its complementary target sequence. The procedure includes the following steps (Figure 1.7): (1) fixation of the specimen; (2) preparation of the sample (may require special pretreatment steps); (3) hybridization with the respective probes to detect the respective target sequences; (4) washing to remove unbound probes; (5) mounting, visualization and documentation of results.

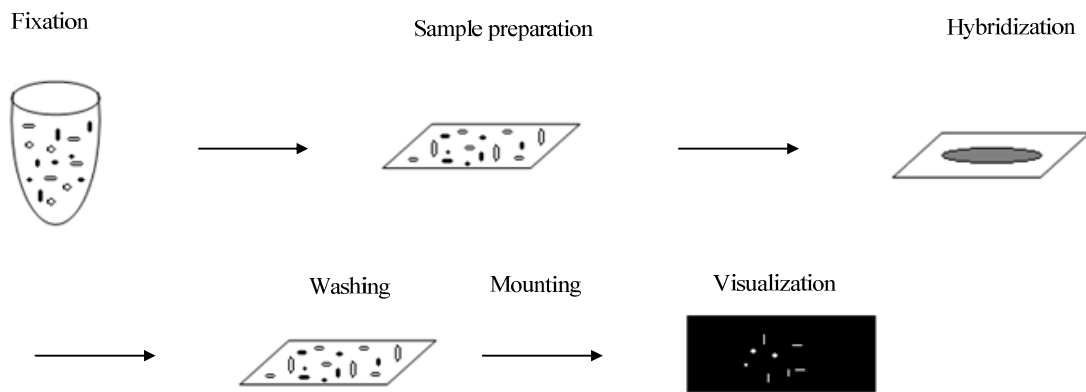


Figure 1.7 A typical FISH procedure

For fluorescence in situ hybridization scheme, it is necessary to label the oligonucleotide probes with fluorophores, immobilize the hybrids on a solid surface, remove unhybridized probes, and then determine the number of probes that remain bound. The requirement that unhybridized probes have to be removed precludes the adaption for real-time monitoring of nucleic acid synthesis and for locating specific nucleic acids in living cells. Furthermore, the need to immobilize hybrids on a solid surface limits sensitivity, since probes bind nonspecifically to surfaces. Although they are suitable for some applications, there are many limitations in plenty of aspects.

1.3.2 Molecular beacon (MB)

Another useful tool for detecting specific nucleic acids in homogeneous solutions is molecular beacon (MB).³¹ Molecular beacons are single-stranded oligonucleotide hybridization probes which form a stem-loop structure. The sequences of the two sides of a MB probe are complementary, so a duplex stem can form by hybridization of the two arms. The loop is comprised of a sequence complementary to a target sequence. A fluorescent dye is labeled to the end of one arm and a quencher is labeled to the end of the other arm. The fluorophore is dimmed by the nonfluorescent quencher when they are positioned close to each other by the stem of the probe in the form of stem-loop structure. However, when a MB anneal to a complementary strand of a specific sequence they form a probe-target hybrid that is longer and more stable than the arm hybrid, forcing it to dissociate. This results the fluorophore and the quencher being away from each other. Therefore fluorescence was restored (Figure 1.8).

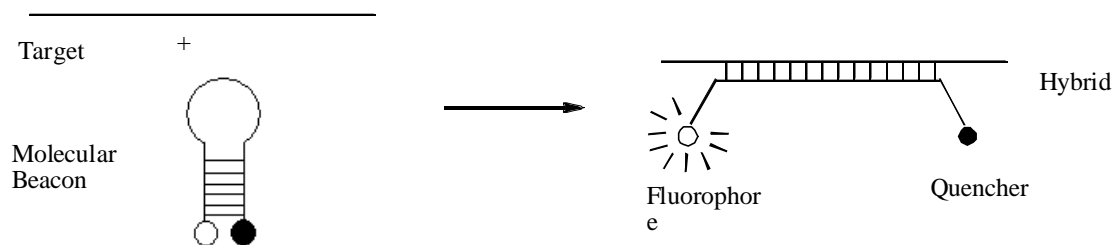


Figure 1.8 Principle of molecular beacons

The hairpin conformation of MB make the use of different colored fluorophores possible.³² By using several molecular beacons, each designed to recognize a different target and each labeled with a different fluorophore, multiple targets can be distinguished in the same solution, even if they differ from one another by as little as a single nucleotide.

In addition, the use of fluorescence resonance energy transfer (FRET) technique could increase the sensitivity of the detection by decreasing the background noise.³³ In a traditional fluorophore-quencher molecular beacon, the same fluorophore absorbs light and emit light of a longer wavelength. In the FRET method, another “donor” fluorophore instead of a quencher, which could interact by FRET with the acceptor fluorophore, is placed a few nucleic acids away from the acceptor fluorophore. When the conformation of this modified molecular beacon is changed by the target, the difference between the excitation wavelength and the emission wavelength (stock shift) is greater than the fluorescence of conventional monofluorophore (fluorophore and quencher) probe.

An important factor in the design of MB probes is the efficiency of energy transfer between the donor and acceptor fluorophore used to label MB probes.³⁴ The FRET efficiency may be strongly influenced by the inclination of the donor and acceptor fluorophore to bind to each other. If multiple MB probes are designed to bind on a target sequence close to one another and to interact by FRET, five nucleotides are the minimum required between each target sequence for each probe, so that contact quenching can be avoided and emission from the acceptor maximized.

The major limitation of MB is the low sensitivity. Since one probe-target hybrid could only give one copy of fluorescence signal, the signal is not amplifiable. When the concentration of the target is low, it might be too difficult to detect the weak fluorescence. The afore-mentioned scorpion probe is one solution to solve this problem. Another isothermal amplifiable MB approach involving exonuclease III is illustrated in a later section.

1.3.3 Template-directed dye-terminator incorporation assay (TDI)

Template-directed dye-terminator incorporation assay is a fluorescence-based technology to detect polymorphic sites.³⁵ A polymorphic site is a chromosome site with two or more identifiable allelic DNA sequences. In this method, a fluorophore-labeled probe, which is designed to bind to the DNA template next to the polymorphic site, is incubated with the amplified genomic DNA fragments with polymorphic sites in the presence of one allelic dye-labeled dideoxynucleoside triphosphates (ddATP, ddGTP, ddCTP or ddTTP) and a modified Taq DNA polymerase. The two fluorophores are a FRET pair. The dye-labeled primer is extended only one base by the dye-terminator specific for the allele present on the template if the ddNTP match the nucleotide at the allelic site. No dye-labeled ddNTP can be incorporated onto the primer if there is a mismatch. At the end of the reaction, without separation or purification, FRET can be detected for positive result (Figure 1.9). This homogeneous DNA diagnostic method is highly sensitive and specific and is suitable for automated genotyping of large numbers of samples.

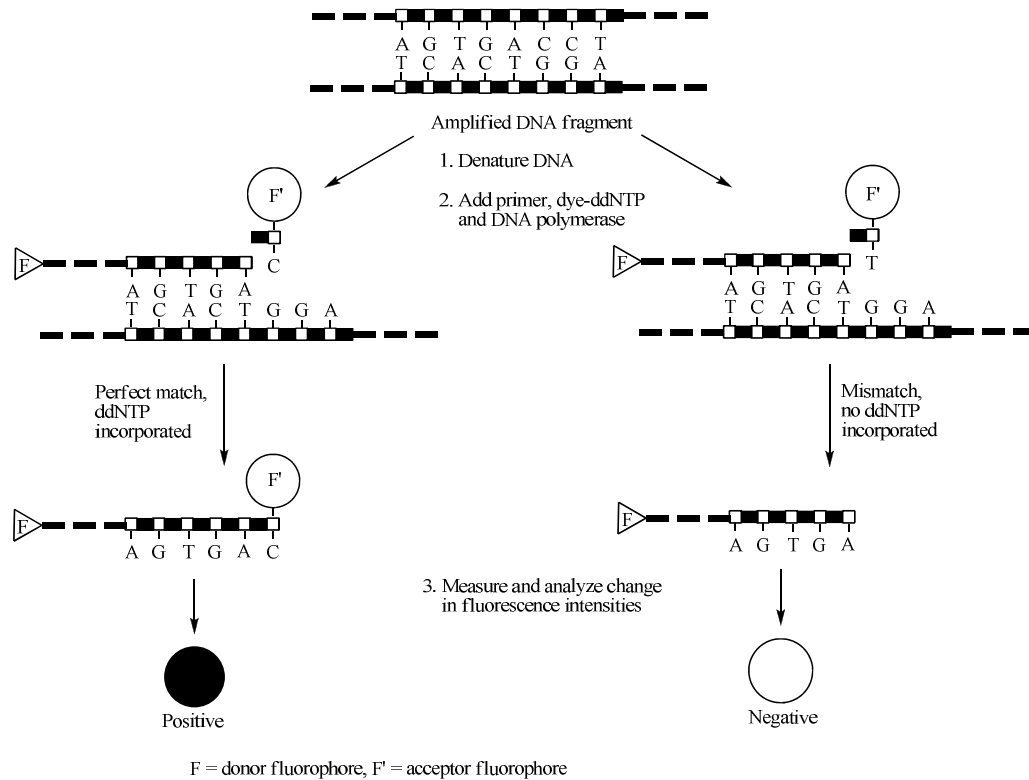


Figure 1.9 Template-directed dye-terminator incorporation assay

The fluorescence signal of TDI can not be amplified so it requires more of the target to get reasonably intense result. Since PCR is required to pre-amplify the target strand, this method can not be applied *in vivo*.

1.4 Amplifiable isothermal nucleic acids detection

1.4.1 Nucleic acid sequence-based amplification (NABSA)

Nucleic acid sequence-based amplification (NASBA)³⁶ is an isothermal amplification method designed to detect RNA target (Figure 1.10). With this method, the forward primer (primer 1 in Figure 1.10) is composed two parts, one of which is complementary to the 3' of the RNA target and the other T7 promoter sequence. At

41 °C, primer 1 binds to the RNA, reverse transcriptase (RT) extends the primer to a complementary DNA (cDNA) of the RNA. RNase H then degrades the RNA strand of the RNA-cDNA hybrid. The reverse primer (primer 2 in Figure 1.10) then binds to the cDNA, and a DNA polymerase produces double stranded DNA (dsDNA), which contains a T7 promoter. After this initial phase, the system enters the amplification phase. The T7 RNA polymerase generates many RNA strands based on the dsDNA, and reverse primer binds to the newly formed RNA. RT extends it and RNase H degrades the RNA of RNA-cDNA during the extension. The newly produced cDNA is a template for primer 1 to bind and the cycle is repeated, leading to several amplification cycles.

NASBA achieves 10 million fold amplification in 1~2 h.³⁷ It is commercialized as detection kits.^{38, 39} A wide range of target RNA sequences were amplified and detected *via* this technique, such as HIV-1 genomic RNA,⁴⁰ hepatitis C virus RNA,⁴¹ Human Cytomegalovirus mRNA⁴², 16S RNA in many bacterial species,⁴³⁻⁴⁷ and enterovirus genomic RNA,⁴⁸ etc. However, NASBA is sensitive and specific to only RNA target, DNA is not a ideal target since RNase H does not digest dsDNA.

DNA polymerase. The newly generated strand still contains a nicking site for HincII. Subsequent nicking of the newly synthesized duplex, followed by DNA polymerase-mediated extension is repeated several times and this leads to an isothermal amplification cascade.

A later generation of SDA used four probes (B1, B2, S1 and S2), instead of two (Figure 1.12).⁵⁰ In the second-generation SDA platform, probes B1 and S1 (or B2 and S2) bind to the same DNA strand (see Figure 1.12). Primers S1 and S2 contain HincII recognition sequences and the four primers are simultaneously extended by DNA polymerase using dGTP, dCTP, TTP and dATP α S. Extension of B1 displaces the S1 primer extension product, S1-ext. Likewise, extension of B2 displaces S2-ext. Extension and displacement reactions on templates S1-ext and S2-ext produce two types of fragments. One has a hemiphosphorothioate HincII at each end and the other has a hemiphosphorothioate HincII site at just one end. HincII nicking and polymerase extension/displacement reactions automatically enter the SDA reaction cycle. This new generation SDA has an expanded scope because it does not require the presence of a restriction endonuclease recognition site in the target of interest.

Strand displacement amplification has been used to amplify *Mycobacterium tuberculosis* genomic DNA (100 to 10⁷ copies) in 2 h.⁵⁰ Like PCR, certain experimental parameters impose some limitations on the procedure.⁵⁰ For example, the amplification capacity decreases as the target length increases, and false positives can be obtained if some strict procedures are not followed.

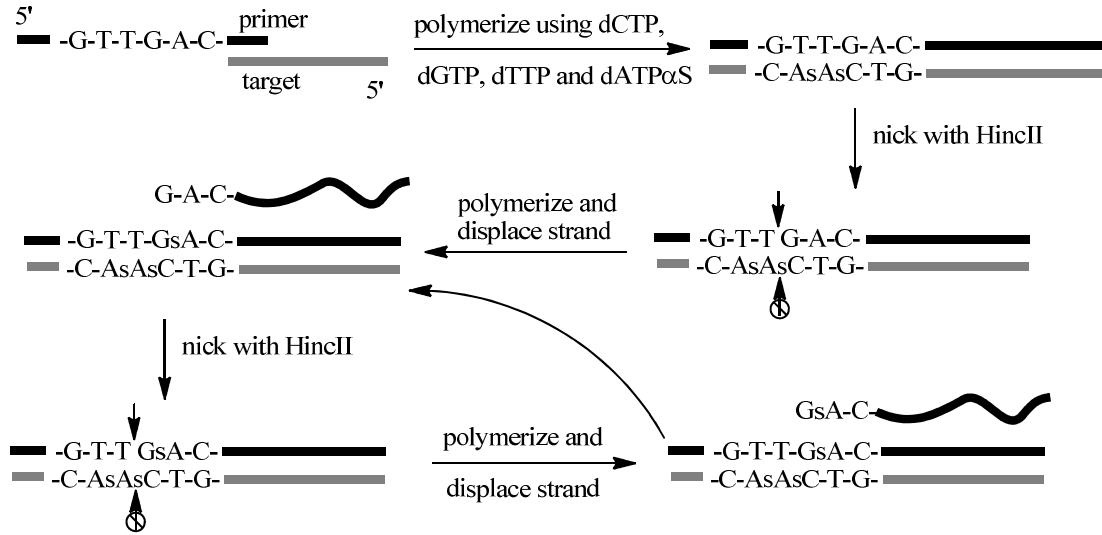


Figure 1.11 First generation strand displacement amplification

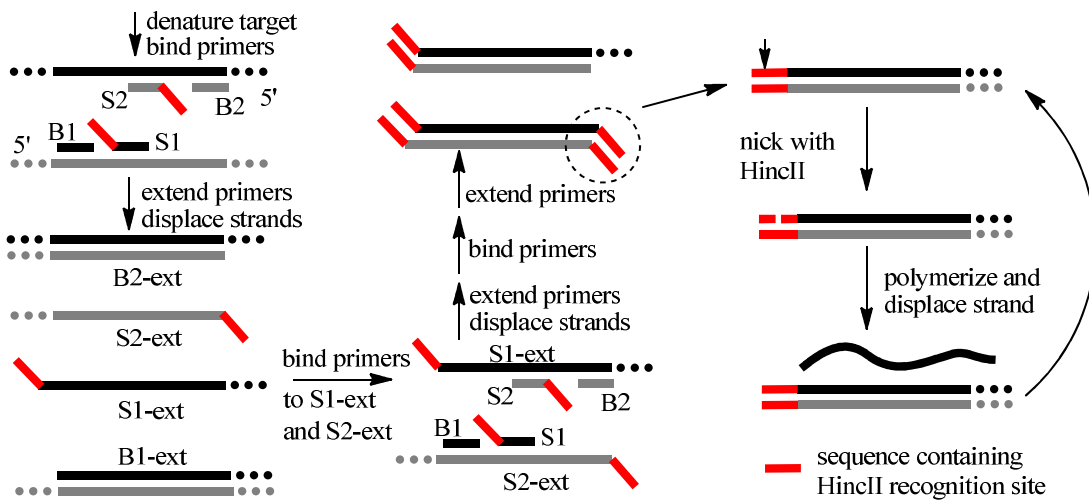


Figure 1.12 Second generation strand displacement amplification

1.4.3 Loop-mediated amplification (LAMP)

Loop-mediated amplification (LAMP) is a highly specific isothermal method to amplify nucleic acids. The high specificity is due to the two sets of primers spanning 6 distinct sequences of the target. The steps of LAMP are illustrated in Figure 1.13. For the initial steps, only the process initiated by the forward primer set is shown for clarity purpose.⁵¹ Two primers in the forward primer set are named inner (F1c-F2, c

strands for “complementary”) and outer (F3) primer. At a temperature around 60 °C, F2 region of the inner primer first hybridize to the target, and is extended by a DNA polymerase. The outer primer F3 then binds to the same target strand at F3c, and the polymerase extends F3 to displace the newly synthesized strand. The displaced strand forms a stem-loop structure at 5’ end due to the hybridization of F1c and F1 region. At 3’ end, the reverse primer set can hybridize to this strand and a new strand with stem-loop structure at both ends is generated by the polymerase. The dumbbell structured DNA enters the exponential amplification cycle and strands with several inverted repeats of the target DNA can be made by repeated extension and strand displacement.

LAMP is accurate and sensitive. It can amplify a few copies of the target to 10^9 in less than one hour regardless of the presence of irrelevant DNA.⁵¹ LAMP has been applied to detect a variety of viral pathogens, including dengue,⁵² Japanese Encephalitis,⁵³ Chikungunya,⁵⁴ West Nile,⁵⁵ Severe acute respiratory syndrome (SARS),⁵⁶ and highly pathogenic avian influenza (HPAI) H5N1,⁵⁷ etc. The major disadvantage of LAMP is the design of the primer sets can be complicated since 6 regions of the target are covered. Also it is not suitable for short nucleic acids sequences such as microRNA.

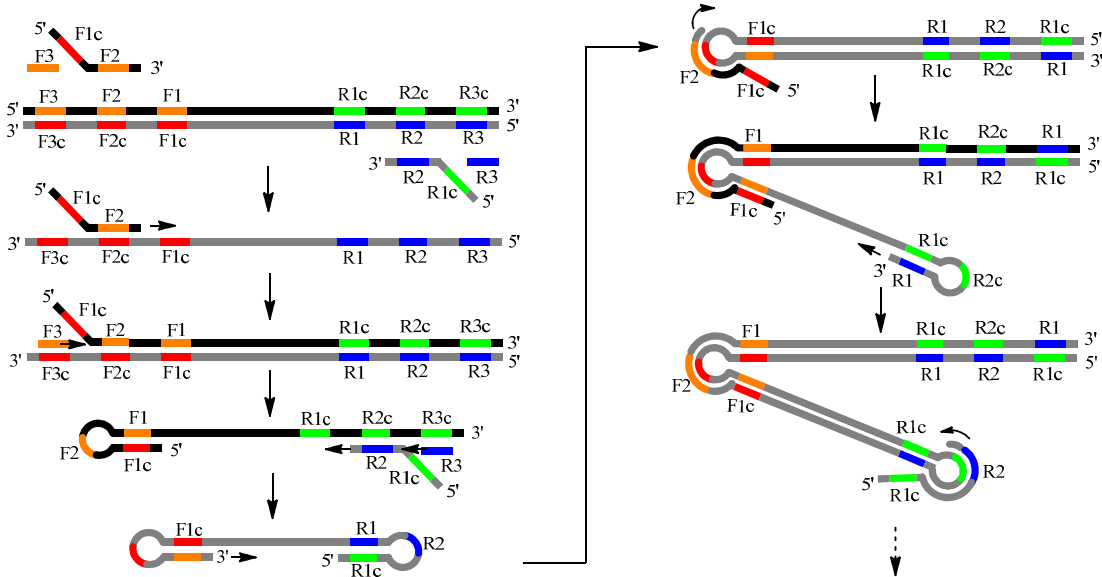


Figure 1.13 Initial steps of Loop-mediated amplification

1.4.4 Invader[®] assay

Two oligonucleotide probes are used in the invader[®] assay.⁵⁸ As illustrated in Figure 1.14, one probe, the Invader[®] oligo, is complementary to the target sequence on the 3' side of the SNP except its 3' ends with a non-complementary base overlapping the SNP site of the target. The other probe, the allele-specific probe extending its non-matching flap 5' end past the polymorphic site, is partially complementary to the target sequence on the 5' side of the SNP and also contains the matched base of the SNP allele (see Figure 1.14, Probe 1). Non-complementary nucleotide at the SNP allele can also be contained in this probe (see Figure 1.14, Probe 2). A three-dimensional invader structure is formed over the SNP site when these two oligonucleotides hybridize to the target strand. A cleavase, a thermostable flap endonucleases (FEN) enzyme, removes excess nucleotides on the bifurcated structure in a structure-specific way and is highly sensitive to sequence mismatches. If both a fluorophore at the 5' end and an internal quencher molecule are attached on

the probe, the cleavage reaction will dissociate the fluorophore from the quencher, as shown in Figure 1.14, and produce a detectable increase of fluorescent signal. If, in contrast, the probe oligonucleotide does not match the SNP allele present in the target DNA (Probe 2), then no cleavage happens. The fluorophore labeled probe is designed to have a melting temperature close to the assay temperature to enable the probe to frequently bind and detach. When the probe binds, the cleavase cleaves it, the remnant detaches, and a new intact probe re-anneals to the same site. This catalytic efficiency of this platform is approximately 3000 cleavages in 90 min per target molecule.⁵⁸

In the described format assay, an initial PCR amplification of the target region is necessary before the invader reaction, since a large amount of targets are required to obtain a measurable signal. Moreover, both of the two distinct allele-specific probes (one for each SNP allele) are needed to be labeled with a fluorophore and a quencher molecule. This adds a significant cost burden to the expense of the method, making it unsuitable for large-scale projects and high-throughput genotyping.

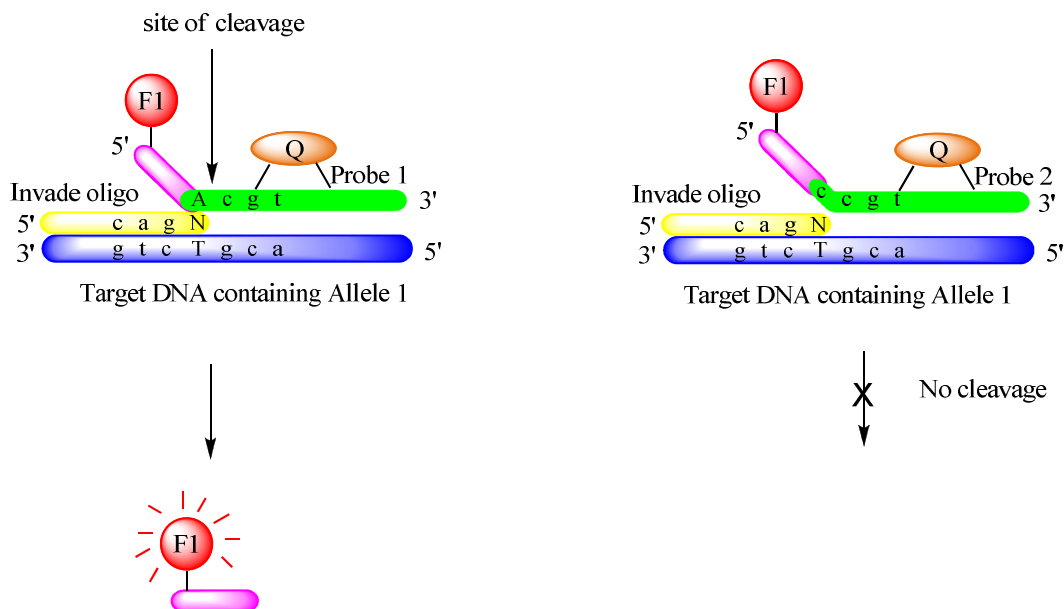


Figure 1.14 Invader[®] assay

One solution to the above difficulties reported by Hall et al. is to combine two invasive cleavage reactions into a single homogeneous assay.⁵⁹ As illustrated in Figure 1.14, the first invasive cleavage reaction is similar to the original reaction. The invader structure is formed by two probes hybridizing with a target molecule. However, one of the oligonucleotides, the primary probe, is no longer labeled with a fluorophore and a quencher on the flap sequence, which includes the base complementary to the SNP allele and is cleaved in the primary reaction. These flap sequences, once released, act as Invader[®] oligonucleotides in a relay invasive cleavage reaction annealing to another FRET cassette (see Figure 1.15). This time, the other oligonucleotide probe with a stem-loop structure, combines both target and primary probe into a single molecule in the three-dimensional invader complex instead of two molecules. The cleavase can cleave one of the stem (as primary probe), separating the fluorophore and the adjacent quencher molecule. This reaction releases the fluorophore and the signal can be measured. Since the flap sequence is not

complementary to the target sequence, it is designed independently from the target sequence. Two generic flap sequences, one for each SNP allele probe, are used in the assay. Each flap sequence is specific for one FRET cassette molecule, and thus generates a distinct fluorescent signal. This assay is advantageous over the previous one because it is significantly more sensitive, and no longer requires the synthesis of allele-specific labeled oligonucleotides. The fluorophore labeled probes, although still not cheap, can be synthesized in large amount and used in any Invader[®] assay regardless of the target sequence.

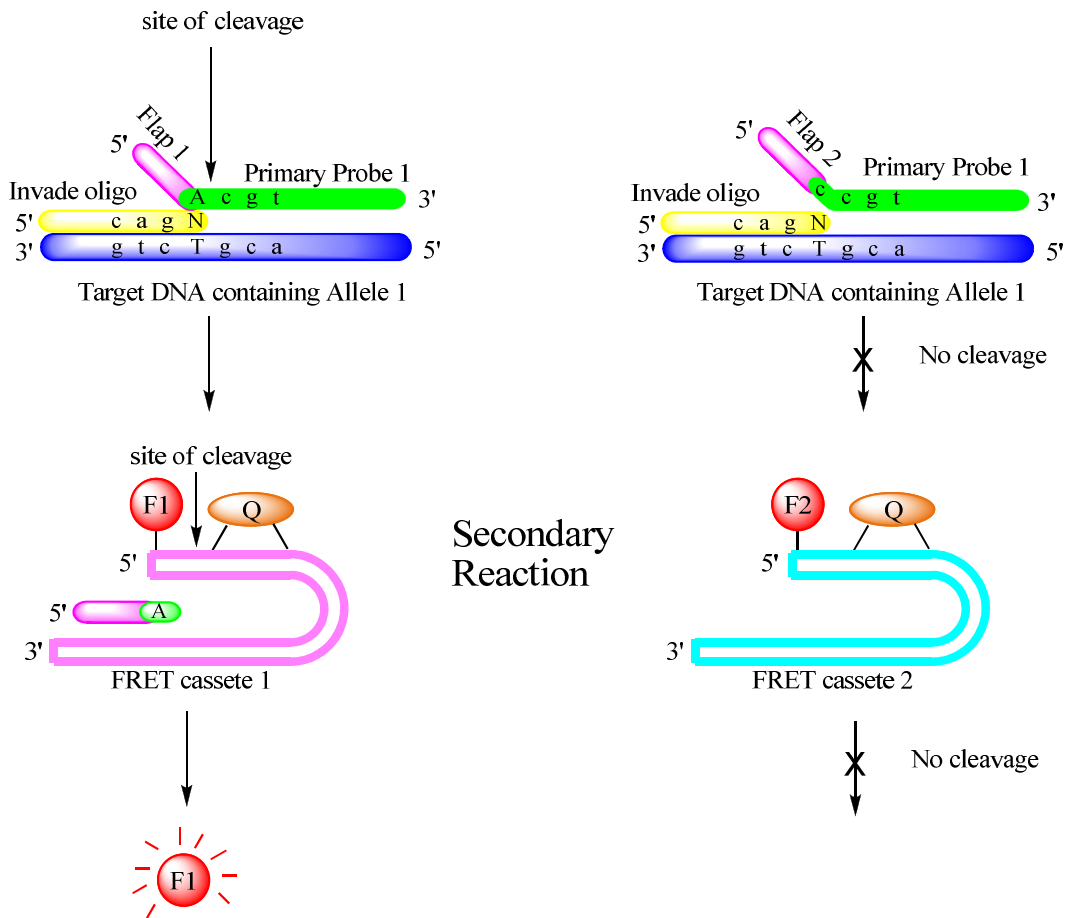


Figure 1.15 Serial invasive signal amplification reaction

Invader[®] assay has a wide range of application, including the detection of SNP of the factor V gene (named Leiden mutation, a well-known hereditary risk factor for

venous thrombosis),⁵⁸ genes responsible for Japanese hearing loss,⁶⁰ human papillomavirus DNA,⁶¹ microRNA from HeLa and Hs578T cells,⁶² and herpes simplex virus types 1 and 2,⁶³ etc.

1.4.5 Padlock probes and rolling circle amplification (RCA)

Two groups independently discovered that DNA polymerase can replicate multiple copies of a sequence taking a circular DNA as a template.^{64, 65} RCA is usually coupled with padlock probe, which is an extension of oligonucleotide ligation assay. The padlock probes comprise two target-complementary end-sequences, designed to hybridize head to tail to a target sequence, joined by a target-non-complementary segment (Figure 1.16). The probes become circularized by the action of a DNA ligase if they hybridize to the correct target sequence.⁶⁶ Next in this RCA process, continuous tandem repeats are generated by a strand displacing DNA polymerase extending a circle-hybridized primer around the circular DNA template (Figure 1.16).^{67, 68} Among its wide application, several disease-related DNAs were detected using this technique,⁶⁹ such as cystic fibrosis transmembrane conductance regulator (CFTR) G542X mutation (leading to cystic fibrosis),⁷⁰ Epstein-Barr virus in human lymphoma specimens,⁷¹ influenza A H1N1 and H3N2 mutations,⁷² porcine circovirus type 2,⁷³ and *Listeria monocytogenes*,⁷⁴ amongst others.

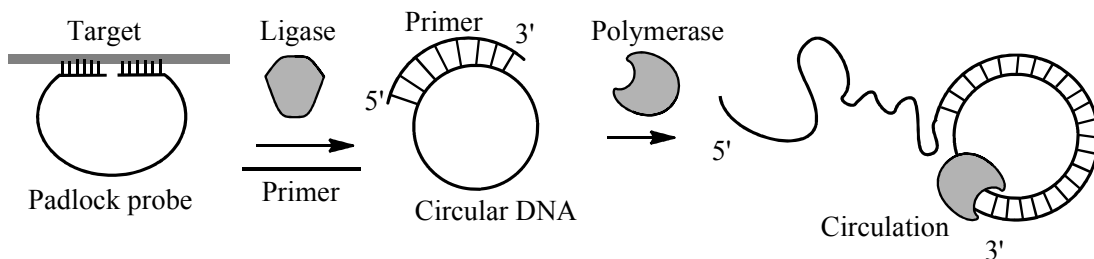


Figure 1.16 Padlock probes and rolling circle amplification

1.4.6 Signal mediated amplification of RNA technology (SMART)

Signal mediated amplification of RNA technology (SMART) is based on the formation of a three-way junction (3WJ) structure. As shown in Figure 1.17, the amplification is initiated by hybridization of the analyte to the two probes. Besides the analyte binding arms, the two probes both contain a part complementary to each other. One of the probes, referred to as the template probe, has a long overhang extending beyond the duplex region between the two probes and its 3'-end is protected from extension with a phosphate moiety. This single overhang contains a T7 RNA polymerase promoter sequence. Once the 3WJ is formed, a DNA polymerase elongates the extension probe along the template probe to produce a double stranded DNA, which is used by T7 RNA polymerase to generate RNA (RNA1 in Figure 1.17). The RNA then can be detected by an enzyme linked oligosorbent assay (ELOSA) or molecular beacon.⁷⁵

SMART is sensitive and can detect 0.05 nM analyte at 41 °C in ~4 hours.⁷⁵ Genomic DNA and rRNA of *E. Coli* can be detected, and even positive signals can be achieved from crude samples.⁷⁵ It has also been applied to detect DNA and mRNA of marine cyanophage virus.^{76, 77}

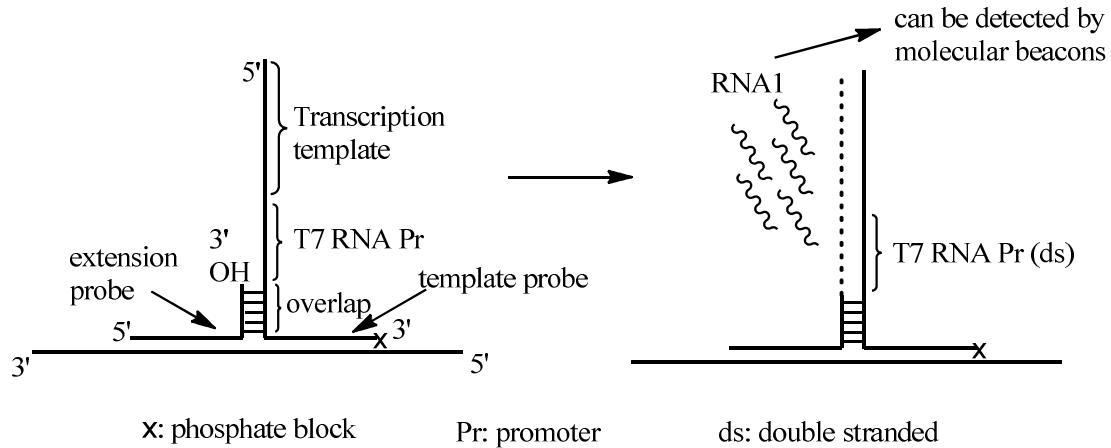


Figure 1.17 Signal mediated amplification of RNA technology

1.4.7 Helicase-dependent amplification (HAD)

Helicase-dependent amplification (HAD) is also a polymerase based isothermal amplification method which uses helicase to separate double stranded DNA instead of heat.⁷⁸ During the amplification, helicases unwind DNA duplexes and single-stranded DNA binding proteins (SSB) stabilize the resulting single strands, which hybridize to primers. DNA polymerase then extends the primers and the newly formed duplexes can act templates for the following reaction cycles (Figure 1.18). The whole process is performed at one single temperature so thermal cycling is not required.⁷⁹ By using a thermally stable helicase, the assay can be performed at 60 °C and this results in improvement in sensitivity and specificity and also eliminates the need for accessory proteins in this assay.⁸⁰ A helicase-polymerase fusion complex was developed and shown to be able to amplify a target of 1.5 kb. The specificity using the helicase-polymerase fusion complex was similar to that obtained using a separate DNA polymerase and helicase.⁸¹

HAD can achieve one million-fold amplification.⁷⁸ A genomic fragment of *Treponema denticola* was amplified in crude bacteria cells and the genomic DNA of the parasite *Brugia malayi* was detected in human blood sample.⁷⁸ However, non-specific amplification has been observed when the concentrations of targets are low in crude samples.⁷⁸

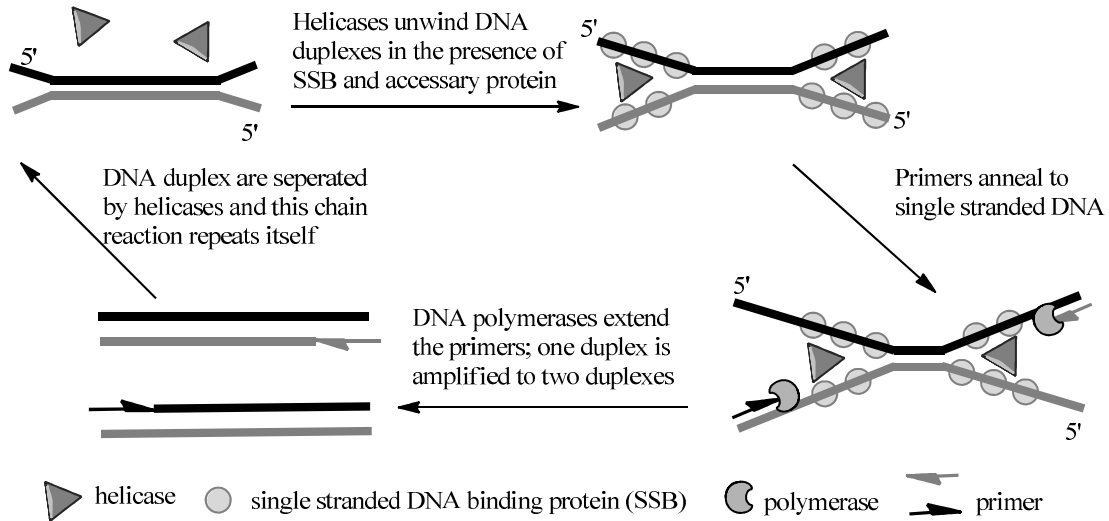


Figure 1.18 Helicase-dependent amplification

1.4.8 Recombinase polymerase amplification (RPA)

In recombinase polymerase amplification (RPA) platform, recombinase-primer complexes initiate the amplification cycle by scanning the duplex template for homologous sequences. Once the specific site is found, the complex opens the double strands and the short oligonucleotides primer is released to bind to the template. The single stranded DNA is stabilized by single-stranded DNA binding protein (SSB) and the polymerase extends the primer. Then the newly synthesized strand displaces the old strand. Exponential amplification is achieved by the cyclic repetition of this process (Figure 1.19).⁸² RPA is fast and sensitive, but background noise can be an

issue. To eliminate background noise, a cleavage step (using an endonuclease) was incorporated at the beginning of the reaction cycle (Figure 1.20). The primer containing a cleavage site for the endonuclease, Nfo (tetrahydrofuran abasic site), is labeled with a fluorophore and a quencher in close proximity on each side of the cleavage site. The cleavage also generates an amplification primer with a 3' OH for DNA polymerase. The endonuclease cleavage step in RPA serves as an additional proofreading step to eliminate background noise.⁸²

Using RPA, three genetic markers, apolipoprotein B, sex-determining region Y and porphobilinogen deaminase from complex human genomic DNA were amplified, and *Bacillus subtilis* genome (only 10 copies) was detected.⁸² The reaction condition for RPA is however stringent and detection using crude samples has not been reported so far.

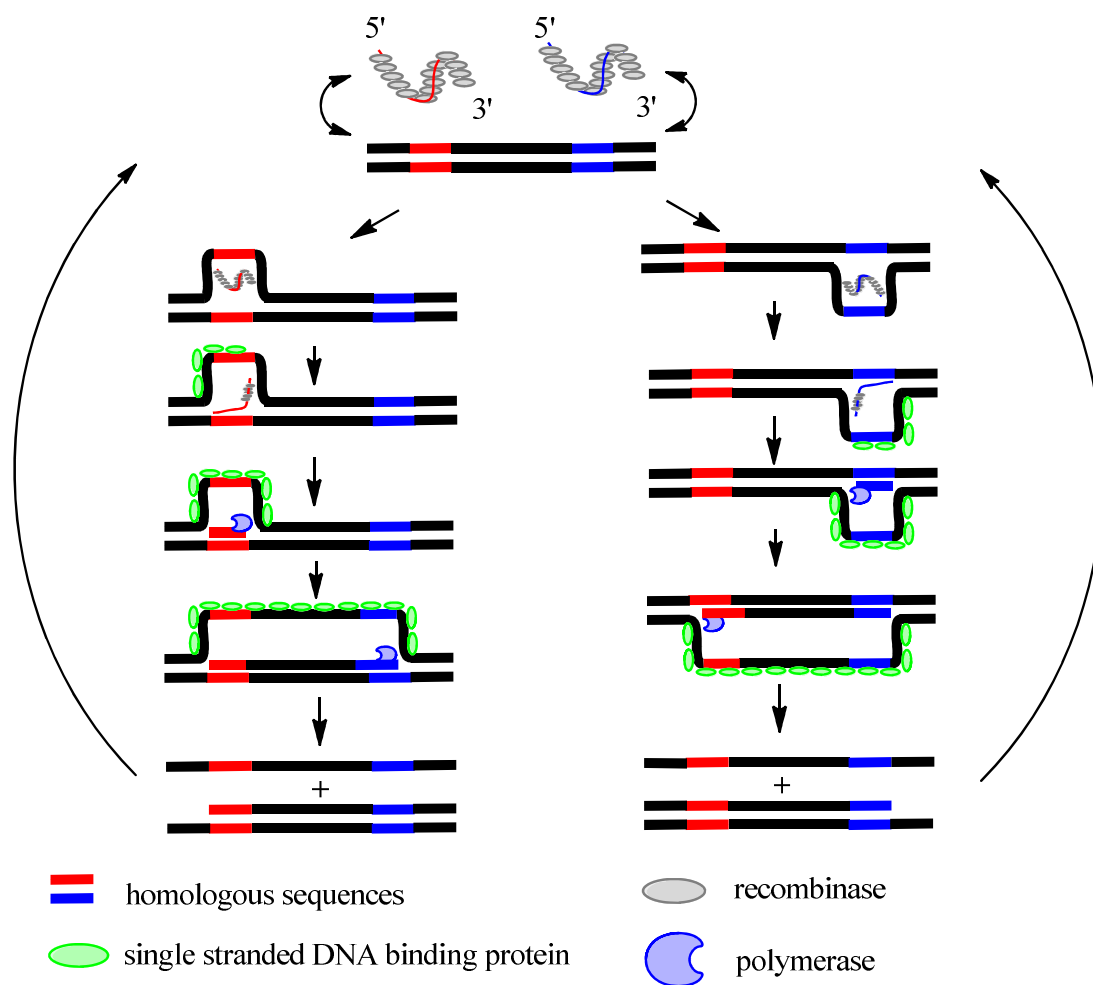


Figure 1.19 Recombinase polymerase amplification

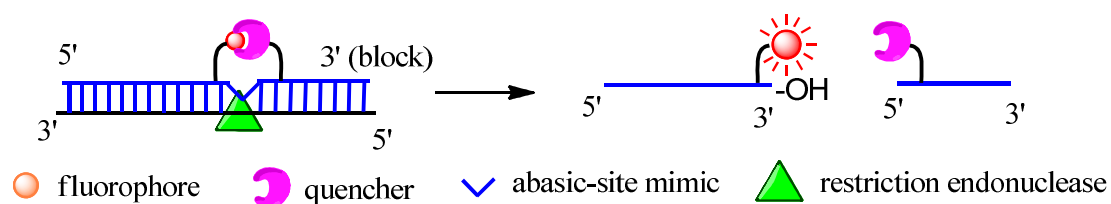


Figure 1.20 Signal generation by separation of a fluorophore and a quencher depends on cutting of the probe by double-strand specific Nfo.

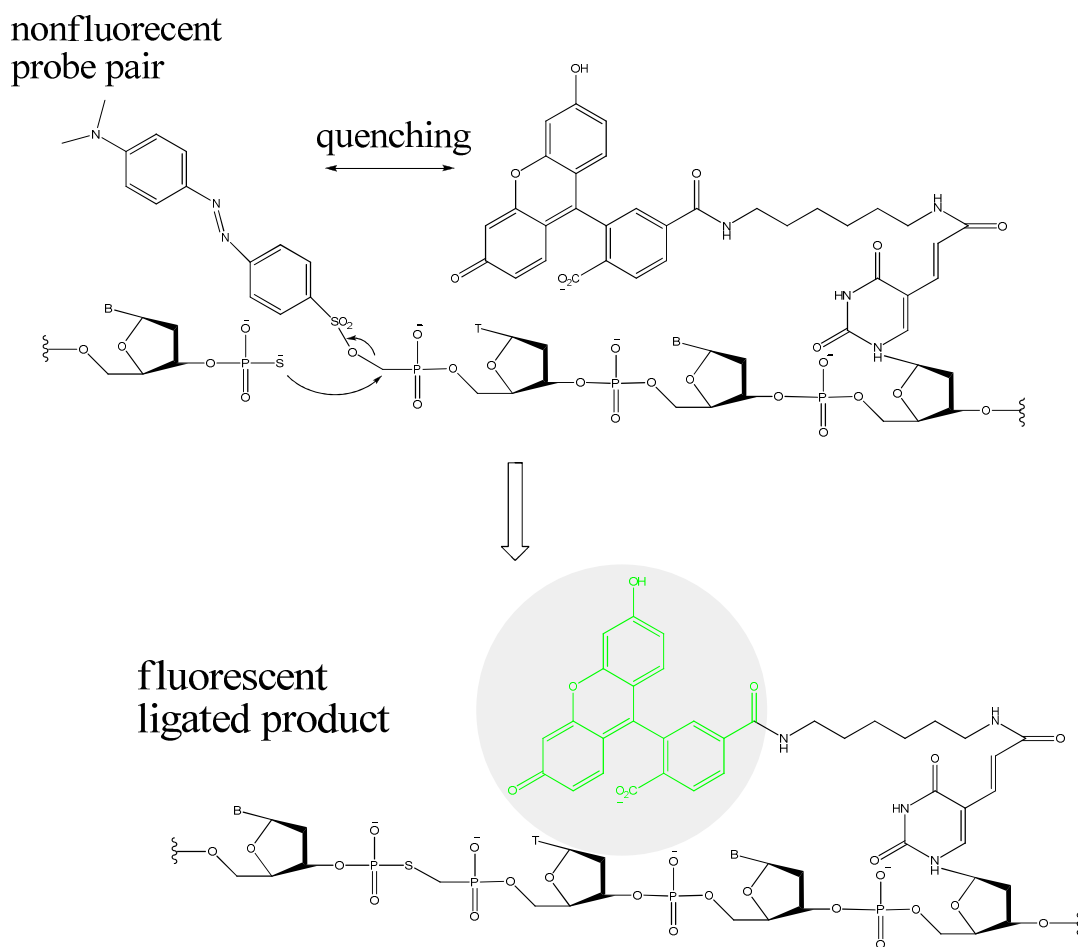
1.4.9 Template-catalyzed chemical reactions

Due to the tremendous progress of chemical modification on nucleic acids, numerous types of DNA template-catalyzed reactions for detection purpose have

emerged in the last twenty years.⁸³ These approaches are divided into two types: chemical ligation-based method and ligation-free signaling. Examples of each type covered here are quenched autoligation – fluorescence energy transfer (QUAL-FRET)^{84, 85} and reporter transfer reaction,⁸⁶ respectively.

In the first case, a pair of probes containing the sequence that is complementary to a target sequence are both modified at their ends. A nucleophile group, e.g. phosphorothioate, is covalently attached to 3'-end of one probe, and 5'-end of the other probe is modified by both a fluorophore and a quenching group. When the probes hybridize to a nucleic target sequence, autoligation (S_N2 reaction) occurs and the quenched group leaves, so fluorescence is restored (Scheme 1.1).⁸⁴ This technology does not need any enzyme. Multiple analytes can be identified by employing different fluorophores attached to the probes.^{87, 88} Like ligase detection reaction, autoligation, as well as other chemical ligations, suffers from product inhibition of template recycling, caused by the increased affinity of longer ligated product compared to the shorter probes. Destabilizing universal linkers for signal amplification self-ligating probes were reported to solve this problem.⁸⁵ The hydrocarbon linkers between the quenching groups and the nucleotides accelerate the ligation reaction. In addition, the hydrocarbon linker destabilizes the duplex, dissociating the ligated product and the target strand. Another pair of probes could hybridize to the target sequence, forming another fluorescent product and the target strand could be reused again (Figure 1.21). Since one target strand gives more than one signal, fluorescence signals are amplified. However, background noise is a major limit. Other nucleophiles, such as amino acids, thiols, and water can react with the

fluorophore labeled probe non-specifically, which initialize a signal emission thus rising background noise and decreasing sensitivity. Moreover, incomplete quenching by dabcy1 is also a source of background. Kool and colleagues attempted to improve the signal to noise by employing FRET.⁸⁹ In this approach, the nucleophile-modified probe is also labeled with a fluorophore-a FRET acceptor Cy5 and the ligation enables the FAM-Cy5 FRET signal. Since neither the target-independent dabcy1 dissociation nor the incomplete FAM quenching adds to the FRET signal, the specificity of QUAL is improved. mRNAs in cells were detected by flowcytometry.⁸⁹ Further improvement in decreasing the assay time was made by changing the phosphorothioate moiety to phosphorodithioate or phosphorotrithioate. These strong nucleophiles shorten the assay time from 3-6 hours to less than 2 hours.⁹⁰ QUAL probes were applied to image in situ rRNA in live cells.⁹¹



Scheme 1.1 Autoligation – fluorescence energy transfer (QUAL-FRET)

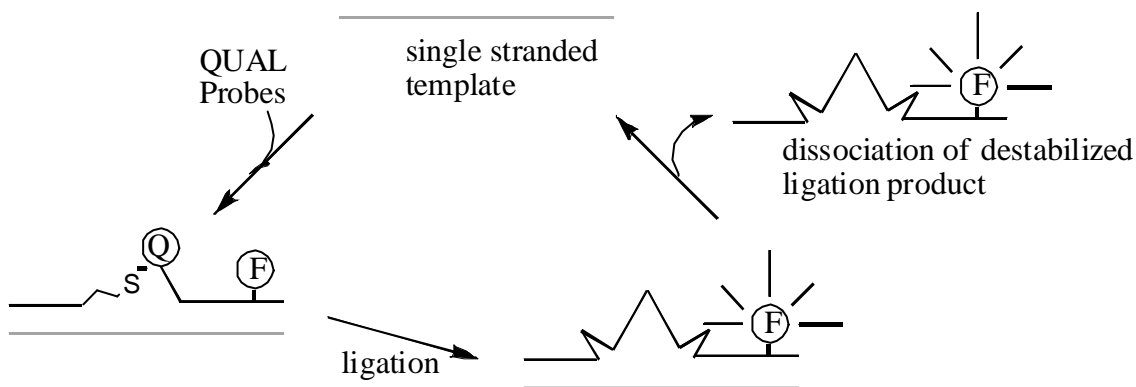


Figure 1.21 Destabilizing linkers for signal amplification in autoligating probes

The reporter transfer reaction detection of DNA circumvents the product inhibition problem by using ligation free process of peptide nucleic acid (PNA) probes.⁸⁶ The

PNA probes are both labeled with fluorophores. The donating probe has a fluorophore (FAM) and nearby quencher (Dabcyl), while the accepting probe has a different fluorophore (TAMRA). When the two probes were positioned in proximity to each other by the analyte, a trans-thioesterification transfers the dabcyl moiety to the probe originally labeled with only TAMRA. The subsequent S→N-acyl shift stabilizes the product, making the reaction irreversible. This dabcyl migration from the donating probe to the accepting probe result in the increase of the fluorescence of the FAM and decrease of that of the TAMRA (Figure 1.22).⁸⁶ Monitoring ratio of $F_{\text{FAM}}/F_{\text{TAMRA}}$ gives a clear indication of the presence of the target. Since the reaction is not a ligation, the affinity of the final products should be similar to the original probes. Dissociation of the reacted probes is required to restore the FAM fluorescence and thus the target can repeatedly catalyze the same reaction cycle by cycle. In an optimized quencher transfer system, 0.02 nM synthetic DNA target was detected with a signal/background ratio of ~1.5.⁹² The method suffers from high background due to the analyte-independent quencher-transfer reaction. Combined with Enzyme-linked immunosorbent assay (ELISA) the quencher-transfer probes were applied to detect HIV genomic RNA.⁹³

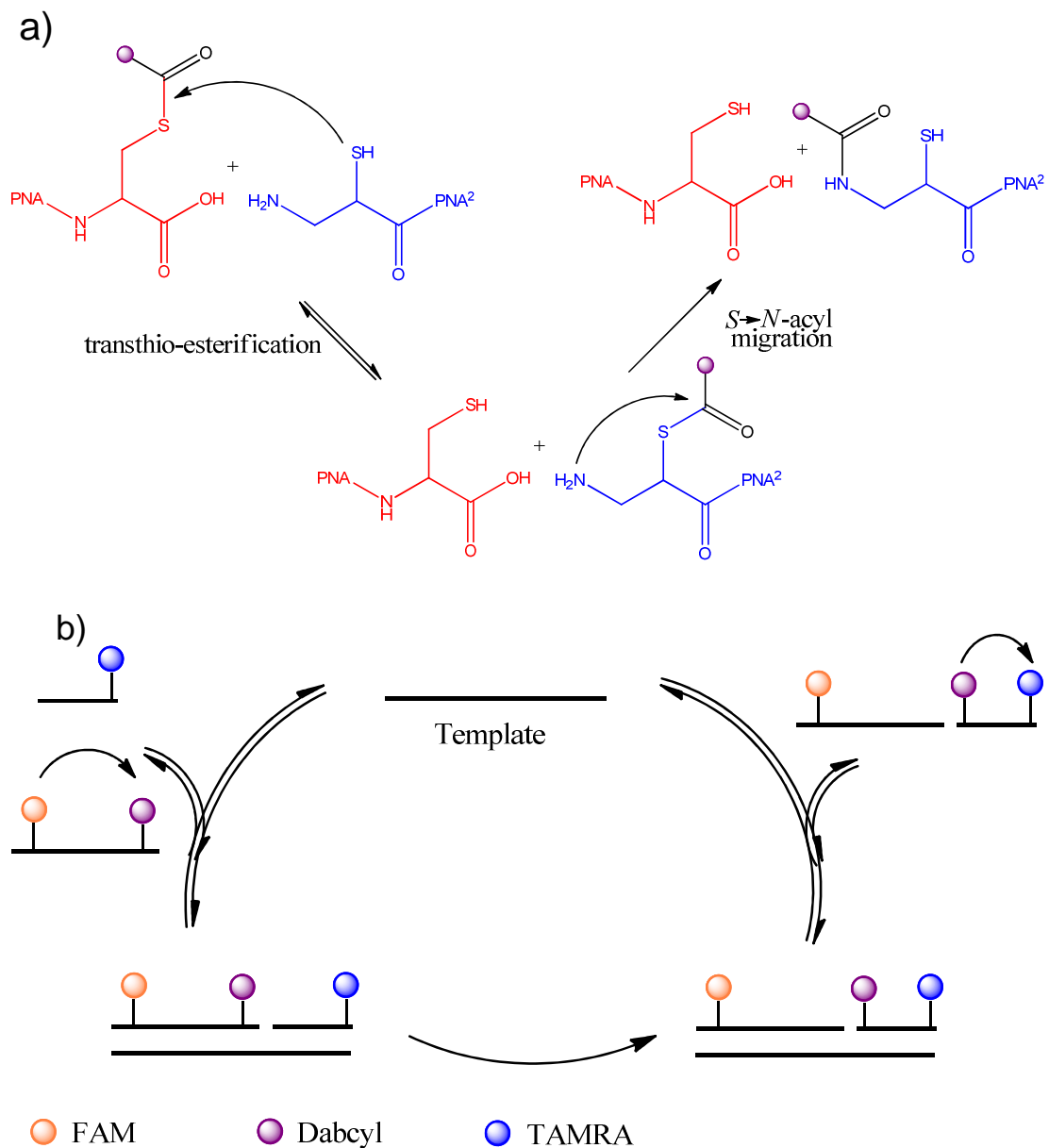


Figure 1.22 Reporter transfer reaction. a) Quencher transfer reaction. b) catalytic cycle of template-catalyzed quencher transfer.

1.4.10 Probe based on RNA cleaving deoxyribozymes

Target-assisted self-cleavage (TASC) probe contains a deoxyribozymes (DNA enzymes, DNAzyme) moiety, which can cleave RNA catalytically.⁹⁴ The probe contains target-binding arms and is labeled with a fluorophore (FAM) and a quencher

(dabcyl) separated by a short nucleic acids sequence embedding a ribonucleotide phosphodiester bond. When the probe hybridize to the target, an active DNAzyme is enabled, leading to self-cleavage, thus separating the fluorophore/quencher pair originally placed on the two sides of the ribonucleotide moiety of the DNAzyme. The two fragments then come off from the target allowing another probe to bind again to drive a catalytic cycle (Figure 1.23).⁹⁵ But the selectivity of this assay is an issue since the DNAzyme can be active even when no target is present. Double mismatched templates were distinguished from the fully mismatched target, but SNP discrimination is not reported.⁹⁵

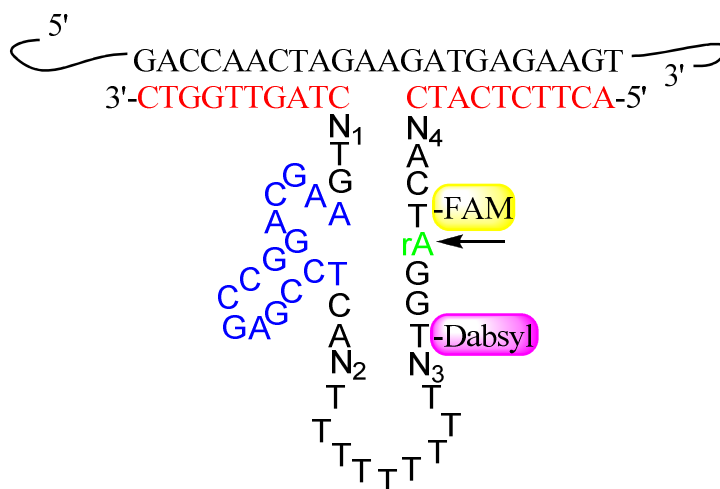


Figure 1.23 Target-assisted self-cleavage probe

In another design based on RNA cleaving DNAzyme, the catalytic core is split into two inactive parts, and the analyte binding arms are linked to each part. The analyte hybridize to the two probes, and active DNAzyme is assembled. A fluorescent substrate is cleaved and fluorescence signal is enabled (Figure 1.24).⁹⁶ Once the enzymatic core is active, fluorescent substrates are cleaved catalytically. In a similar design based on a more efficient DNAzyme, ~0.2 nM analyte was detected after 3 h

of incubation.⁹⁷ Coupling with other non-linear amplification platforms may further lower the limit of detection.^{98,99}

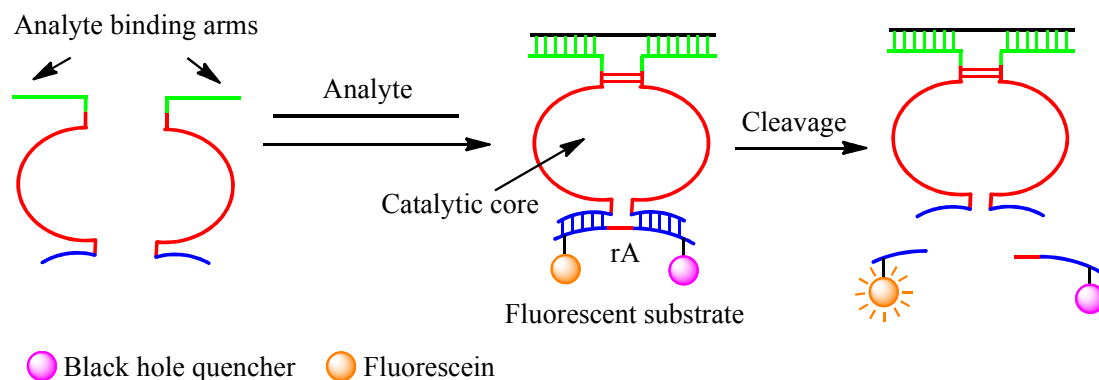


Figure 1.24 Binary deoxyribozyme probes

The advantage of the later enzyme-free assays is their *in vivo* application¹⁰⁰ (or their potential application *in vivo*) due to the fact that fewer components need to be delivered into the living cells.

1.4.11 Split peroxidase-like DNA enzyme probes

A hemin binding G-quadruplex DNA acting like peroxidase¹⁰¹⁻¹⁰³ was incorporated in the design of split peroxidase-like DNA enzyme probes (Figure 1.25).¹⁰⁴⁻¹⁰⁶ In this strategy the two probes contains two regions: analyte binding arms and split peroxidase parts. The splitting pattern can be even¹⁰⁴ or uneven.^{105, 106} When the two probes are separate, they show no or very low peroxidase activity. When the target hybridizes to both of the two probes, the G-quadruplex peroxidase-like DNAzyme is assembled. The hemin-binding complex can oxidize ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) with H_2O_2 and the color change of reaction solution can be monitored visually or by UV instrument (Figure 1.25a). Sintim group recently reported that the introduction of a stem-loop or loop-stem-loop motif

connecting the G3-tracts in a G-quadruplex structure can effectively reduce the background noise (signal generated in the absence of target gene) and subnanomolar target could be detected (Figure 1.25b).¹⁰⁶

Split peroxidase-like DNA enzyme probes assay was applied for the detection of PCR amplified DNA from *Salmonella* and *mycobacterium*.¹⁰⁷ The assays are fast (reactions complete in minutes) and convenient (visually detectable signals are produced), but it is limited by the detection of nanomolar analyte.

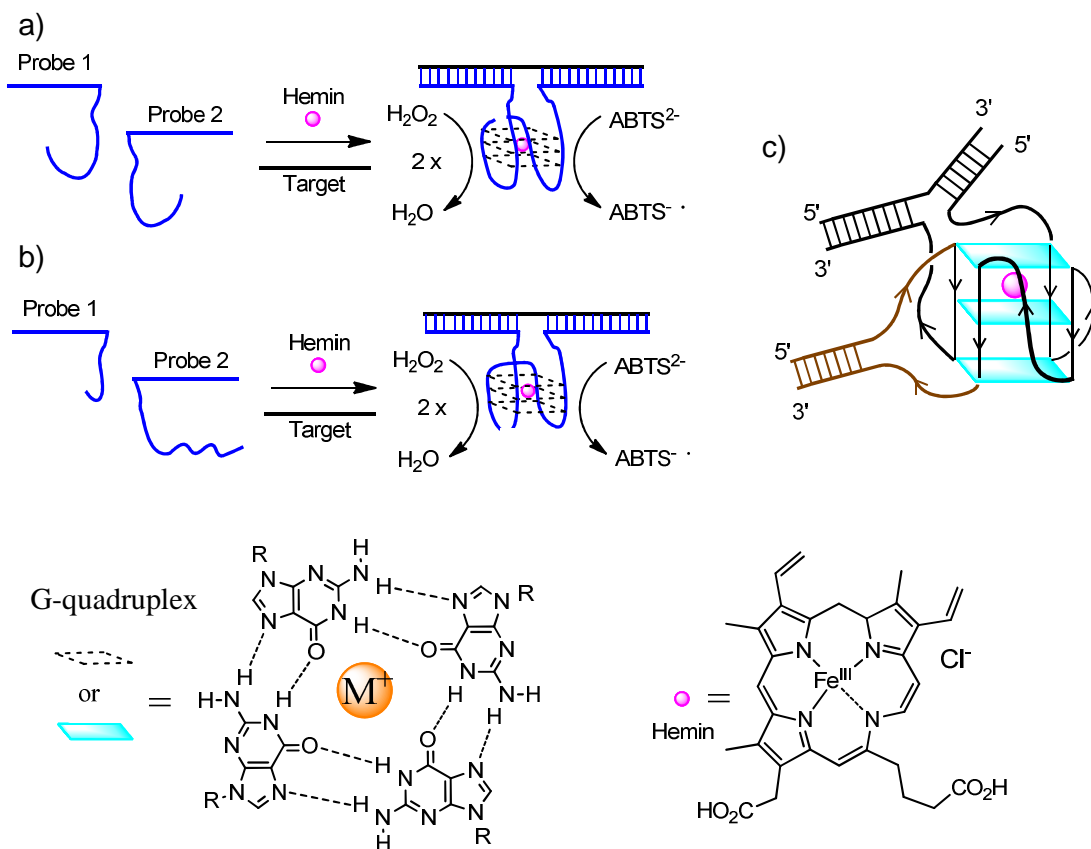


Figure 1.25 Split peroxidase-like DNA enzyme probes. a) Evenly split DNAzyme probes. b) Unevenly split DNAzyme probes. c) Optimized split DNAzyme probes.

1.4.12 Nicking endonuclease assisted amplification (NEAA)

Other than exonuclease, several groups have developed amplification detection of nucleic acids detection based on nicking endonucleases.¹⁰⁸⁻¹¹⁰ Nicking endonuclease usually do not cleave single strand DNA, but recognize double strand cognate site and nick only one strand. In one colorimetric assay by Liu et al., a linker specifically designed to be cleaved by the nuclease anneals to the target. The cleaved products resulting from the nicking of the enzyme fall off from the target, which can be used many times by other linkers. When the reaction finishes, two sets of gold nanoparticles with sequences partially complementary to the linker were added to the solution. If the linker was a mismatch and not cleaved, three-component sandwich complex can form because nanoparticles aggregate, enabled by DNA hybridization (Figure 1.26).¹⁰⁹ Despite the simplicity of this assay, the requirement of nicking endonuclease cognate site in the target sequence precludes the possibility of detecting all target sequence.

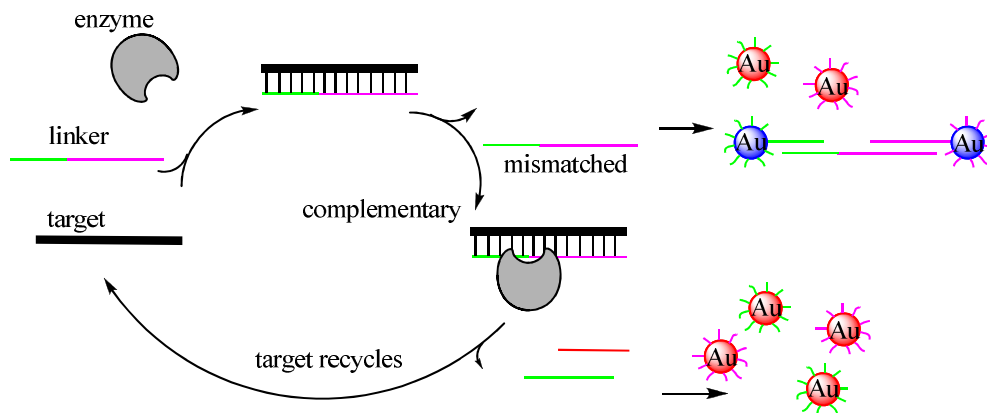


Figure 1.26 Nicking endonuclease assisted nanoparticle amplification

1.4.13 Exonuclease III aided target recycling approach

As mentioned before, traditional MB only produces one signal per probe since one probe occupies one target after binding. Plaxco et al. provided a signal-amplification MB method under isothermal condition by introducing exonuclease III into the system.¹¹¹ Exonuclease III non-specifically digests double strands DNA, rarely single strand DNA, from blunt 3' termini. The stem-loop probe containing a nuclease resistant site between the fluorophore and quencher is designed to anneal to the target to form a structure with one blunt 3' termini. The nuclease cleaves the probe stepwise at 37 °C since the probe exposes its blunt 3' end. Fluorescence increases due the separation of the fluorophore and quencher, and thus the target is released, free to participate in more cycles (Figure 1.27). This method is simple and sensitive enough to detect as low as 20 aM analyte (but requires long assay time-24 hours). Also it is not suitable for RNA analyte because the exonuclease does not cleave DNA-RNA hybrid.

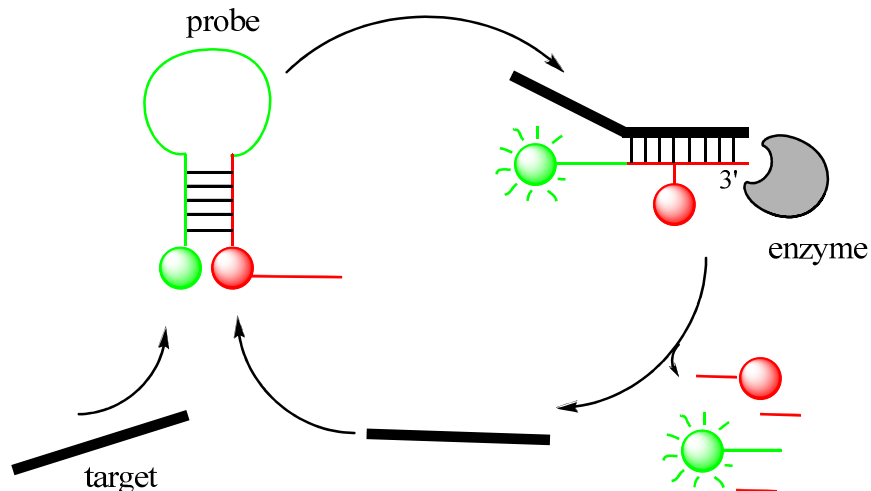


Figure 1.27 Exonuclease III aided target recycling approach

Table 1.1 Summary of isothermal amplification/detection platforms

Platform	Pros	Cons	Components	Application
NABSA	10 ⁷ fold amplification in 2 h, commercial kits available	Not ideal for DNA analyte	2 primers, reverse transcriptase, RNase H, RNA polymerase, dNTP, rNTP	HIV-1 genomic RNA, ⁴⁰ hepatitis C virus RNA, ⁴¹ Human Cytomegalovirus mRNA ⁴² , 16S RNA in many bacterial species, ⁴³⁻⁴⁷ and enterovirus genomic RNA, ⁴⁸ etc.
SDA	10 ⁵ fold amplification in 2 h	Less efficient on long target, sample preparation is required	4 primers, DNA polymerase, REase HincII, dGTP, dCTP, dTTP, dATPαS	<i>Mycobacterium tuberculosis</i> genomic DNA ⁷⁸
LAMP	~10 ⁸ amplification in less than 1 h in the presence of interfering sequences	Complicated primer design, not suitable for short analyte	4 primers, DNA polymerase, dNTP	viral pathogens, including dengue, ⁵² Japanese Encephalitis, ⁵³ Chikungunya, ⁵⁴ West Nile, ⁵⁵ Severe acute respiratory syndrome (SARS), ⁵⁶ and highly pathogenic avian influenza (HPAI) H5N1, ⁵⁷ etc.
Invader [®] assay	Commercial kits available, ideal for SNP genotyping	Initial PCR required	2 or 3 probes, FEN cleavase	the factor V gene, ⁵⁸ genes responsible for Japanese hearing loss, ⁶⁰ human papillomavirus DNA, ⁶¹ microRNA from HeLa and Hs578T cells, ⁶² and herpes simplex virus types 1 and 2, ⁶³ etc.

RCA	Up to 10 ¹² fold amplification in 1 h, multi-target detection simultaneously	Not ideal for RNA analyte,	1 probe and ligase for padlock probes detection, 1 primer, polymerase, dNTP for amplification	cystic fibrosis gene, Epstein-Barr virus in, ⁷¹ influenza A H1N1 and H3N2 mutations, ⁷² porcine circovirus type 2, ⁷³ and <i>Listeria monocytogenes</i> , ⁷⁴ etc.
SMART	50 pM analyte detected in ~4 h, tolerate crude sample	Two-step process	2 probes, DNA polymerase, RNA polymerase, dNTP, rNTP	DNA and rRNA of <i>E. Coli</i> , ⁷⁵ DNA and mRNA of marine cyanophage virus ^{76, 77}
HDA	10 ⁶ fold amplification in 3 h, commercial kits available	Complicated buffer optimization, expensive	2 primers, helicase, SSB, DNA polymerase, dNTP	<i>Treponema denticola</i> gene, ⁷⁸ <i>Brugia malayi</i> gene ⁷⁸
RPA	100 copies of analyte were detected in 1 h, selective	Stringent reaction condition	2 primers, recombinase, SSB, DNA polymerase, REase Nfo, dNTP	<i>Bacillus subtilis</i> genome ⁸²
Template-catalyzed chemical reactions	Simple, no enzyme needed	High background	2 primers	16S RNA of <i>Escherichia coli</i> , ⁸⁴ HIV genomic RNA ⁹³
Probe based on RNA cleaving deoxy-ribozymes	Simple, no protein enzyme needed	Not selective	1 or 3 probes	N/A ^a
Split peroxidase-like DNA enzyme probes	Simple, no protein enzyme needed, fast, colorimetric	high detection limit (nanomolar)	2 probes, hemin	<i>Salmonella</i> and <i>mycobacterium</i> ¹⁰⁷

NEAA	Simple, colorimetric		Requirement of certain recognition sites in analytes	1 probe, nicking endonuclease, gold nanoparticle	N/A ^a
Exonuclease III aided target recycling approach	Simple, 20 aM detected		Not suitable for RNA	1 probe, exonuclease III	N/A ^a

a. These methods are relatively new; hence application to clinical targets is not available.

Chapter 2: First generation of junction probe

Despite the abundance of the described nucleic acids detection methods in chapter one, several limitations of the current state-of-the-art detection platforms have encouraged the development of alternative platforms. Sintim Group is in the process of developing a new detection method. The goals include:

1. Establishing a fluorescent assay that obviates the need for thermal cycling.
2. Detecting DNA target of picomolar concentration.
3. Distinguishing DNA alleles with SNP.

2.1 Introduction of restriction endonuclease

This chapter deals with the use of restriction endonucleases for nucleic acids sequences detection. Restriction endonucleases (REases) are components of restriction modification (RM) systems, which bacteria use to defend themselves against bacteriophage and other external DNA.^{112, 113} This is done by destroying foreign DNA as it enters the cell. Bacteria protect their own DNA via the methylation (N4 or C5 at cytosine or N6 at adenine) within the REase recognition site. Restriction enzymes are categorized into four types.¹¹⁴ Type I restriction enzymes recognize two asymmetrical sequences separated by 6-8 nucleotides and cut at sites that are away (usually kilobases) from their recognition sites. The cofactors S-Adenosyl methionine (AdoMet), adenosine triphosphate (ATP) and Mg^{2+} are required for type I REases.¹¹⁵ Type III restriction enzymes recognize two separate non-palindromic sequences in a head-to-head fashion, translocate the DNA in an ATP-hydrolysis dependent manner, and cut DNA 20-30 base pairs after the recognition site.¹¹⁶ Cofactors ATP and Mg^{2+}

are needed for the cleavage reaction. Type IV restriction enzymes recognize two recognition sequences with methylated or hydroxymethylated cytosine in one or both strands, and the cleavage happens randomly close to one of the recognition sites. Cofactors GTP and Mg^{2+} are necessary for DNA cleavage.¹¹⁷

Type II restriction enzymes are the most abundant type amongst the four types and more than 3500 Type II restriction enzymes have been reported (see restriction endonuclease database for a list and more detailed information - <http://rebase.neb.com/rebase/rebase.html>). They differ from Type I, III and IV in many ways. For example, they usually do not require AdoMet or NTP for cleavage – Mg^{2+} is the only necessary cofactor.¹¹⁸ Many of them are homodimers or homotetramers.¹¹⁸ Because of the great diversity among Type II restriction endonucleases, this large family is divided into several subtypes, which are defined using a letter suffix (Table 2.1).

Table 2.1 Subtype of Type II REases

Subtype ^a	Define feature	Examples	Recognition sequences ^b
A	Asymmetric recognition sequence	Bpu101	CC↓TNA-GC GG-ANT↓CG
B	Cleave both sides of target on both strands	Bp1I	(8/13)GAGN ₅ CTC (13/8)
C	Cleavage and modification domains in one polypeptide	BcgI	(10/12)CGAN ₆ TGC(12/10)
E	Two copies of recognition sequence: one cleaved, one allosteric effector	EcoRII NaeI	↓CCWGG GGWCC↓ GCC↓CGG CGG↓GCC

F	Homotetramers, two copies of recognition sequence, both cleaved co-ordinately	Cfr10I NgoMIV	R↓CCGG-Y Y-GGCC↓R G↓CCGG-C C-GGCC↓G
G	Cleavage and modification domains in one polypeptide, stimulated by AdoMet	Eco57I	CTGAAG(16/14)
H	Similar to Type I gene structure	AhdI	GACN ₂ -N↓N ₂ GTC CTGN ₂ ↓N-N ₂ CAG
M	Subtype of IIP or IIA, require methylated target	DpnI	G ^{m6} A↓TC G ^{m6} A↓T ^{m4} C C--T ↓AG ^{m6} CT ^{m4} ↓AG ^{m6}
P	Symmetric recognition and cleavage sites	EcoRV	GAT↓ATC CTA↓TAG
S	Cleavage site outside of the recognition sequence	FokI	GGATG(9/13)
T	Heterodimers	BsII	CCNN-NNN↓NNGG GGNN↓NNN-NNCC
Nicking	Top strand cleavage	Nt. BstNBI ^c Nt. CviQXI	GAGTCNNNN↓ R↓AG

a. Note that not all subtypes are mutually exclusive, e.g. BcgI (in bold) is the archetype of the Type IIC class, but is also in subtypes IIA, IIB, IIF, IIG and IIH.

b. In cases where cleavage occurs outside the recognition sequence, the sites of cleavage are noted by two numbers: the first indicates the number of bp between recognition and cleavage sites in the strand shown and the second in the complementary strand. Cleavage sites within recognition sequences are marked by ↓. N, any nucleotide; Y, a pyrimidine; R, a purine.

c. Nt stands for Nicking enzyme with top strand cleavage activity.

All Type II restriction endonucleases need to find their recognition sequences in the abundance of nonspecific sequences, to which they can also bind with

considerably lower affinity.¹¹⁹ Three mechanisms are proposed to explain the target site locating process by DNA-binding proteins: (i) 'sliding'; (ii) 'jumping'; and (iii) intersegment transfer.¹²⁰⁻¹²² In sliding mode, the search starts with a nonspecific binding of protein to DNA. It then stays bound and moves along the DNA until it finds the specific site or dissociates (Figure 2.1a). Jumping indicates a three-dimensional mode in which the protein constantly binds and unbinds the DNA, moves to another sites or even another molecule, and repeats the cycle (Figure 2.1b). This mode of diffusion appears less efficient than sliding. Intersegment transfer can be involved only if the proteins have two DNA binding sites. For example, a type IIE restriction enzyme cleaves one specific sequence and the responsible subunit is released, but the other subunit still remains bound to the DNA with the other site. A different specific site on the same molecule can bend and bind to the protein, in which loops can form (Figure 2.1c). It is assumed that both sliding and jumping contribute to the target site search,¹²³ but the extent involved depends on the conditions such as Mg^{2+} concentration and ionic strength.^{120, 124}

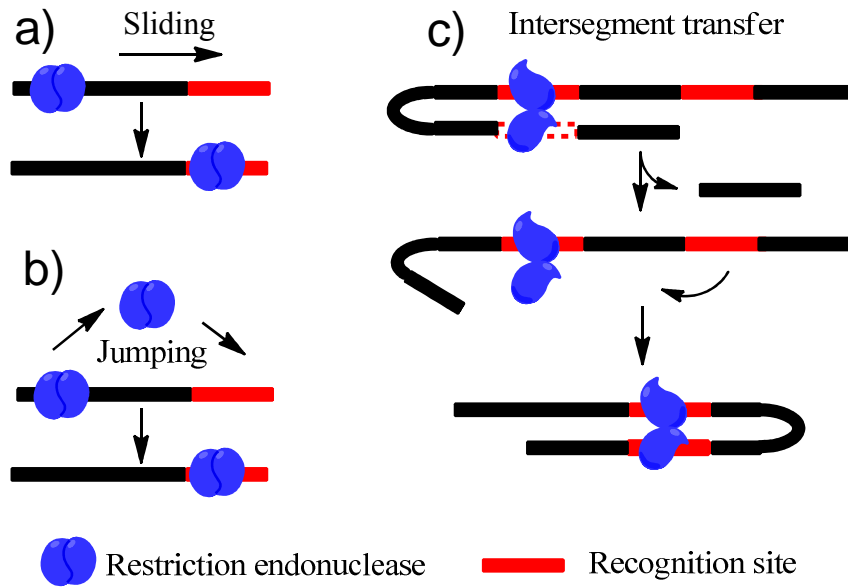


Figure 2.1 The recognition site search movement modes of REase. a) Sliding; b) jumping; c) intersegment transfer.

The recognition process usually triggers the conformational change of both enzymes and DNA. In a recognition event, the distortion of DNA brings its functional groups into the positions for optimal interaction with the enzyme, and the phosphodiester bond to be hydrolyzed is also placed to the catalytic center. The enzyme undergoes conformational changes involving structure transformations of its subunits and subdomains. Accompanied with these more or less pronounced adaptations, a hydrogen bond network is formed. The hydrogen bonding is major component in the contact patterns between enzymes and DNA. The bases as well as the sugar-phosphate backbone can participate in the hydrogen bonds formation, directly with the enzyme or mediated by water. For example, the crystal structures of HinP1I¹²⁵ (pre-reactive complex and post-reactive complex) show that the 10 bp DNA duplexes substrate undergo protein induced distortions, including bending, base flipping and minor groove expansion. In the case of the enzyme HinP1I, the β -strand

and the loop connected to a helix αA become an extension of the helix upon binding to cognate DNA. HinP1I interact with the phosphate backbone, spanning 6 bp, 2 of which are outside of its recognition site G↓CGC. And all 8 bases of the recognition sequence form direct hydrogen bonds with one HinP1I molecule (Figure 2.2).

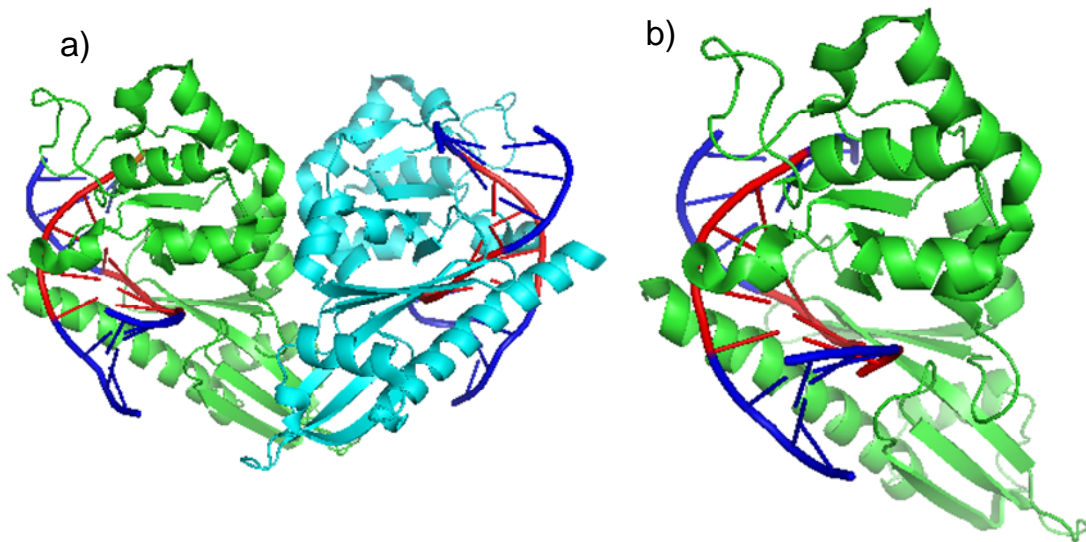
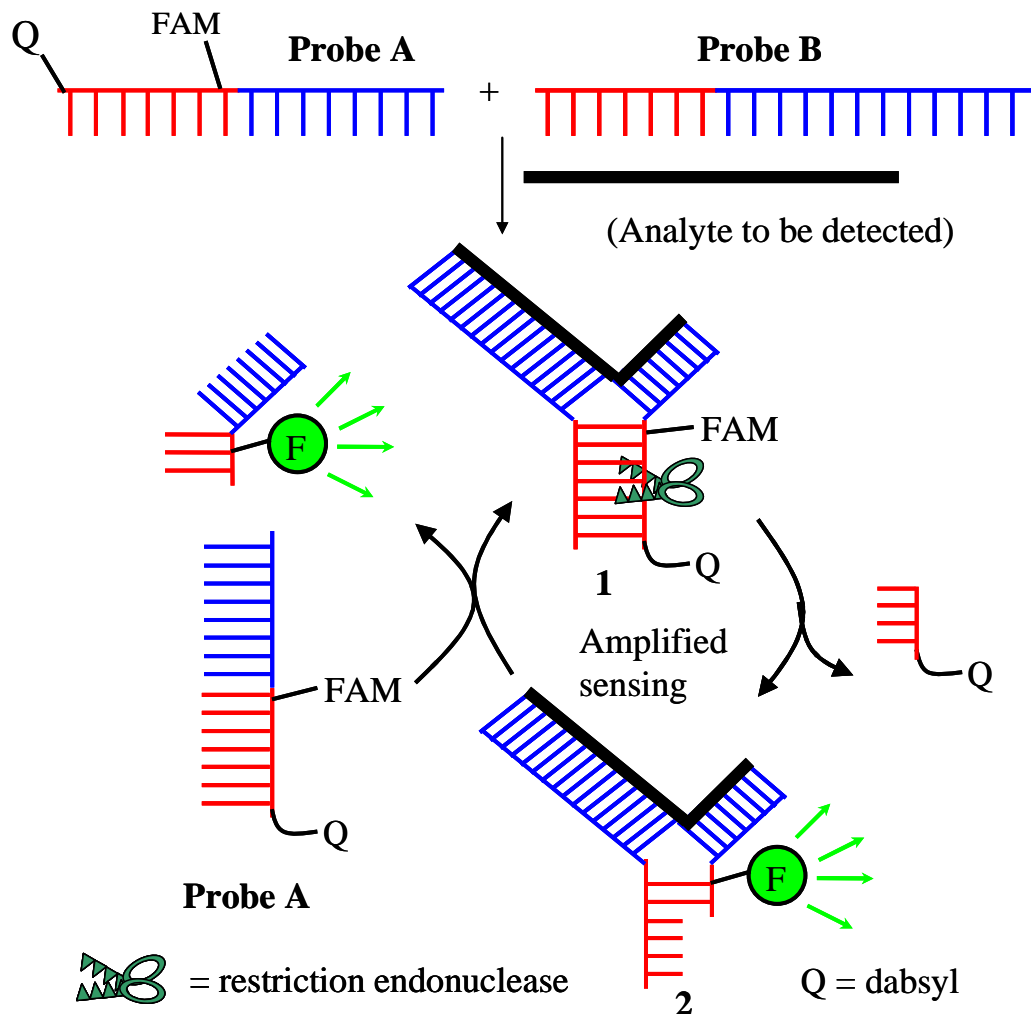


Figure 2.2 Crystal structures of HinP1I with cognate DNA. a). Pre-reactive complex. b) Post-reactive complex.

2.2 Concept of junction probe via template enhanced hybridization process

Recently, there has been an interest in the use of type II restriction endonucleases (REases) for the detection of both DNA and RNA in clinical samples. For example, several researchers have described the detection of DNA and RNA as well as amplification and processing of DNA nanostructures utilizing various endonucleases.¹²⁶⁻¹³² REases are important DNA modifying enzymes and over 3500 have been discovered to date. However, DNA detection assays at a constant temperature (isothermal condition) that utilize these enzymes are lacking because of

the requirement for a particular recognition sequence in target analytes if REases are to be used for ultra-sensitive DNA detection. The new nucleic acid detection technology Sintim group is developing is called junction probe (JP) technology,¹³³ which has provided an elegant means to eliminate the need for a particular REase recognition site in target, and also achieved isothermal amplified sensing *via* a concept called Template Enhanced Hybridization Processes (TeHyP). In TeHyP strategy, two probes that do not hybridize to each other at a certain temperature were designed to anneal to each other in the presence of a template *via* the formation of a ternary structure (Scheme 2.1). The resulting complex that forms after the template enhanced hybridization can then be detected by various means/processes (see Scheme 2.1 for an example).



Scheme 2.1 Amplified sensing using TeHyP concept

In our first generation JP method, one of the probes, A, is labeled with a fluorophore (fluorescein - FAM) and a quencher (Dabsyl). The FAM and Dabsyl molecules are separated by several nucleotides (colored red in Scheme 2.1) that contain a REase cognate site. Since REases only cleave double-stranded DNA, single strand probe A can not be cleaved by REases and the FAM fluorescence is quenched by Dabsyl in proximity. Probe A and the unlabeled probe B are both composed of two parts: a region containing the REase site and complementary to each other (red), and the other region complementary to parts of the DNA/RNA target sequence (colored

blue in Scheme 2.1). The melting temperature of the short duplex that results from probe A annealing to probe B is lower than 15 °C. Therefore, it was expected that in the absence of a template that can enhance the hybridization of probe A and B, probes A is not able to be cleaved by REases because it will predominately exist as single-stranded at temperatures around 30 °C. However, in the presence of a complementary template, probes A and B and the template hybridize to form a ternary “Y” junction structure, complex **1** (see Scheme 2.1). One pre-programmed arm of the Y junction structure which contains a cleavage site for a REase can be subsequently cleaved by this enzyme, ripping the quencher away from the fluorophore. Thus the fluorophore “lights up” and an increase of fluorescence can be detected. The cleavage also results in another ternary structure **2** which has a lower stability than complex **1**. Another intact probe A can replace the cleavage product from the previous probe A for a next reaction cycle.

2.3 “Y” junction and REases

Three-way DNA junctions can be cleaved by some Type IIS restriction endonucleases,¹³⁴ which cleave at sites separated by precise number of nucleotides from the recognition sequence. However, the recognition site was in one arm and the cleavage site was in another arm (e.g. REase FokI, see Figure 2.3). These enzymes are not suitable for junction probe development because if such enzymes were used, the design would require the recognition site in the target sequence. To the best of our knowledge, there has not been no other detailed studies on how other type II REases operate on the three-way junction.

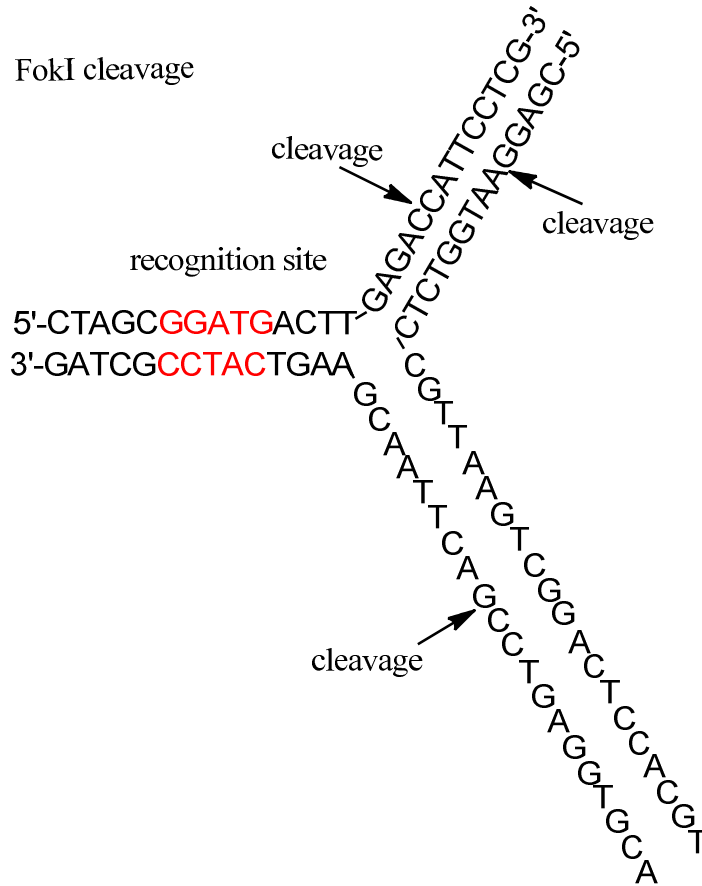


Figure 2.3 Three-way DNA junction cleavage by Type IIS REase FokI

The orthodox Type II Reases, Type IIP (P for palindromic) enzymes which usually recognize palindromic sequences of 4-8 base pairs (bp) in length and cleave both strands within these sequences, are candidate enzymes for junction probe. In the design of junction probe, the affinity of the complementary regions of probe A and B should be kept to a minimum to avoid probes A-B duplex formation without the presence of target, i.e. duplex **D1** in Figure 2.4, since that may result in background enzymatic cleavage. Thus category 1 REases in Table 2.2 were not chosen since recognition sites of 5 and more bp may cause a long complementary region of probe A and B. Despite their 4 bp recognition sites, category 2 REases were also avoided due to their GC-rich recognition sequences since G-C base pair has stronger hydrogen

bonding than A-T base pair. So category 3 REases are preferred since the recognition sites of 4 bp are not GC rich.

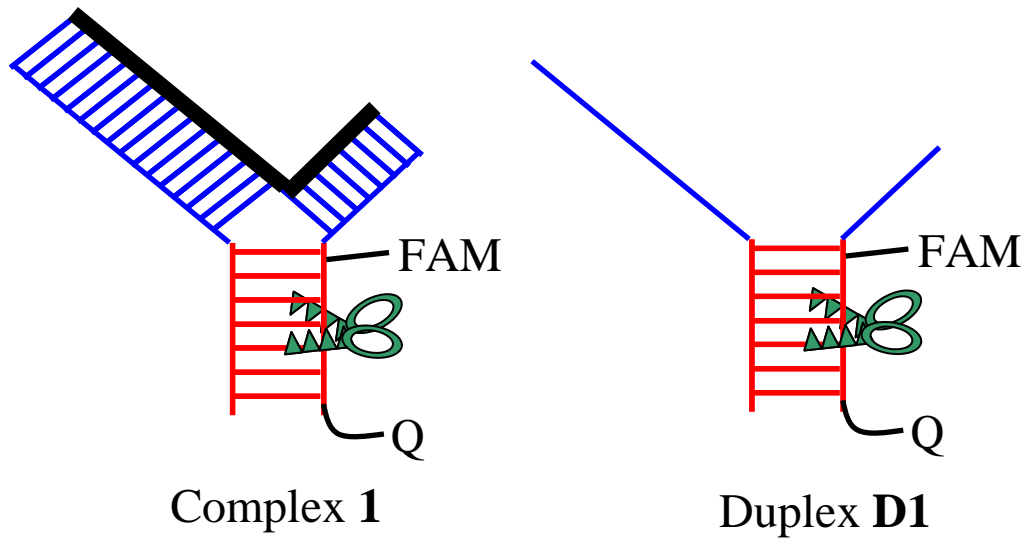


Figure 2.4 Y junction complex 1 and duplex D1

Table 2.2 Examples of Type IIP restriction endonucleases

Category	Feature	Enzyme examples	Recognition sites ^a
1	Recognition sites of 5 bp or more	ZraI	GAC↓GTC
		AsiSI	GCGAT↓CGC
		NheI	G↓CTAGC
		PdmI	GAANN↓NNTTC
		DriI	GACNNN↓NNGTC
		Swal	ATTT↓AAAT
2	GC rich recognition sites of 4 bp	HaeIII	GG↓CC
		MspI	C↓CGG
		BstUI	CG↓CG
		HhaI	GCG↓C
		HinPII	G↓CGC
3	GC not rich recognition	BfuCI ^b	↓GATC

sites of 4 bp	FspBI	C↓TAG
	MluCI	↓AATT
	MseI	T↓TAA
	NlaIII	CATG↓
	CviQI	G↓TAC

a. The “↓” indicates the point of cleavage. N: any nucleotide

b. BfuCI has many commercially available isoschizomers including DpnII, MboI, Sau3AI, and NdeII.

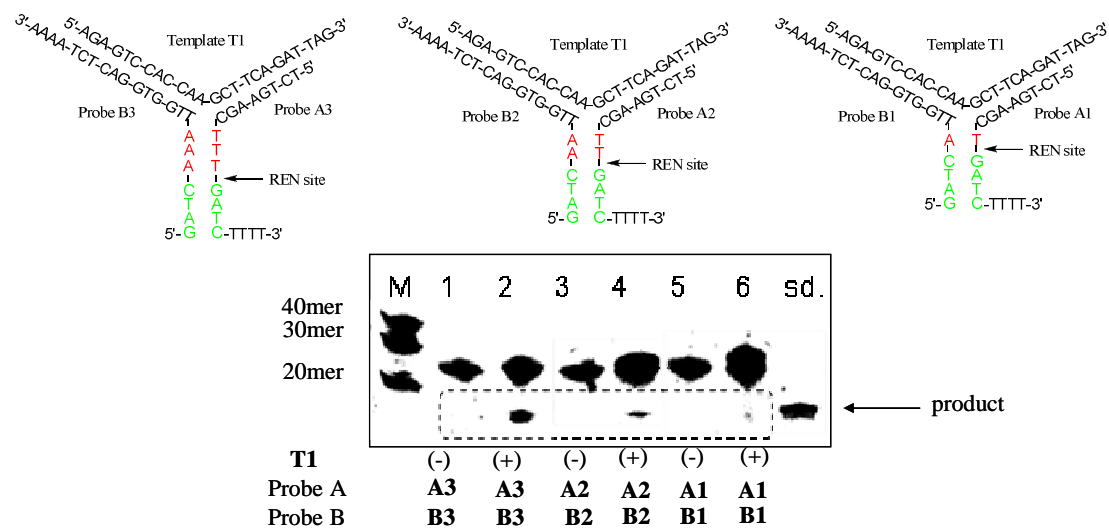
2.4 The influences of flanking sequences on REases activities

Although the influence of a junction to REase activity is not studied thoroughly, a different factor, the flanking sequences of the recognition sites of REases, has been investigated to some extent. These studies were done to shed more light on the observation that a restriction enzyme can cleave their cognate site at rates that depended on the sequences of the flanking sequences to the recognition sites.¹³⁵ The differences in cleavage rates were suggested to originate from how the bases flanking the target site affect endonuclease binding or the cleavage event. The activities of EcoRI,¹³⁶ PstI (5'...CTGCA/G...3'),¹³⁷ and HinfI (5'...G/ANTC...3')¹³⁷ could be inhibited by long flanking runs of dG and dC. It is suggested that an altered helix conformation at runs of dG and dC was carried over into the enzyme binding site so as to disturb its structure.¹³⁸ A more detailed examination of the enzymes FnuDII (5'...CG/CG...3'), HaeIII (5'...GG/CC...3'), HhaI (5'...GCG/C...3') and MspI (5'...C/CGG...3') revealed 7~20 fold difference of cleavage rates due to the flanking

bases variation.¹³⁹ However, the effects were complicated and no general patterns were concluded.

2.5 Sequence length between the junction and the REase site

We started with a category 3 REase BfuCI to explore our junction probe platform. One reason of this choice is that there are many commercially available isoschizomers (enzymes recognize the same sequence and cleave at the same site); hence one set of probes could be used to screen different enzymes. As mentioned before, it is important to keep the region of sequence complementarities between the two detection probes to a minimum in order to avoid a template independent hybridization and a subsequent REase cleavage that will lead to high background noise. For the restriction endonuclease enzyme, BfuCI, 3 base pairs are preferred between the REase recognition site and the junction in complex **1** since they gave best yield while keeping the background low (Figure 2.5).



M: DNA Marker; sd.: 5'-CTAATCTGAAGCTTT-3' (15mer).

Figure 2.5 Sequence length between the junction and the REN site. Reaction conditions: [probe A] = [probe B] = [template] = 2 μ M, [BfuCI] = 0.067 U/ μ L, buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [dithiothreitol (DTT)] = 1 mM, [bovine serum albumin (BSA)] = 100 μ g/mL. 30 $^{\circ}$ C, 5 h.

It is likely that at the junction, localized melting results in the formation of a bubble. This might account for the low cleavage rate observed when there is only one base pair between the cleavage site and the branch point. In this case, the cleavage site might be partially melted.

2.6 Fluorophore position between the junction and the REase site

We observed that for the REase, BfuCI, the distance between the fluorophore and the cognate site was crucial for the efficient cleavage at the cognate recognition site. When FAM was placed on the thymine nucleotide that lied next to the recognition site or two nucleotides away from the REase recognition site (5' end), no cleavage

product was observed. However, when FAM was moved three nucleotides away from the site, the cleavage product could be obtained (Figure 2.6). It is possible that when FAM was close to the REase cleavage site, the aromatic moiety of FAM either intercalated into the REase recognition site and retarded cleavage or blocked the enzyme from latching onto the recognition sequence.

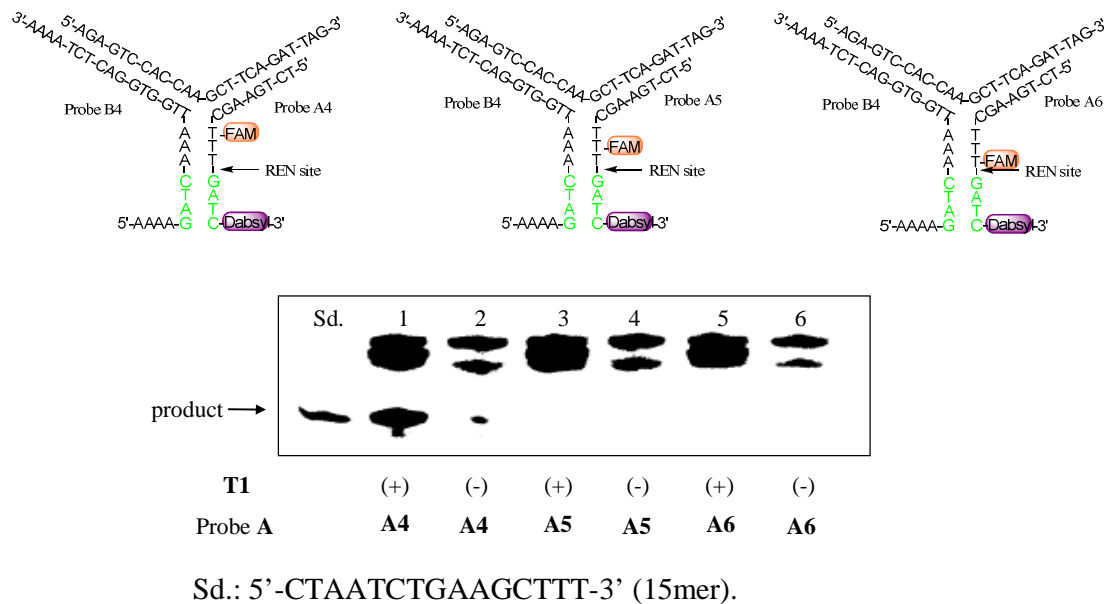


Figure 2.6 Fluorophore position between the junction and the REase site. Reaction conditions: same as those for Figure 2.5.

2.7 Homogeneous fluorescence detection of DNA target

Homogeneous fluorescence detection of DNA target can be achieved by monitoring the increase of the fluorescence of the JP reaction. Time course fluorescence experiments showed that the rate of cleavage at the REase 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** (Figure 2.7). The difference between them was readily distinguishable in one hour.

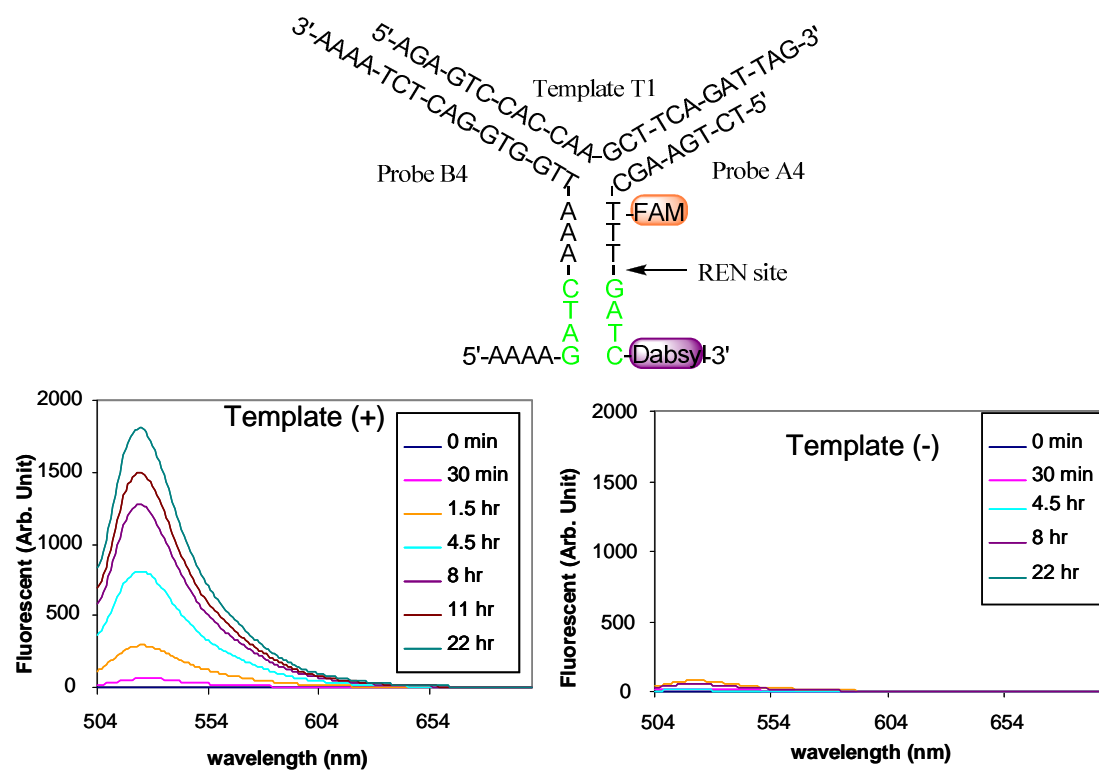


Figure 2.7 Homogeneous fluorescence detection of cleavage product. Reaction conditions: [probe A] = [probe B] = [template] = 1 μM , [BfuCI] = 0.0267 U/ μL , buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [DTT] = 1 mM, [BSA] = 100 $\mu\text{g/mL}$. 30 °C.

2.8 SNP detection

Template detection by junction probes is also sequence selective. We monitored the reactions using both gel and fluorimeter. Single mismatch targets (**T2-T4**) were used as templates and the mismatch site was placed in region of the template, where probe A binds (colored blue in the table of Figure 2.8). The result showed the sensitivity of JP detection to a single mismatch. 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 2.8a). Similarly, the fluorescence intensities obtained with single mismatch targets were similar to that of the target-free reaction (see Figure 2.4b).

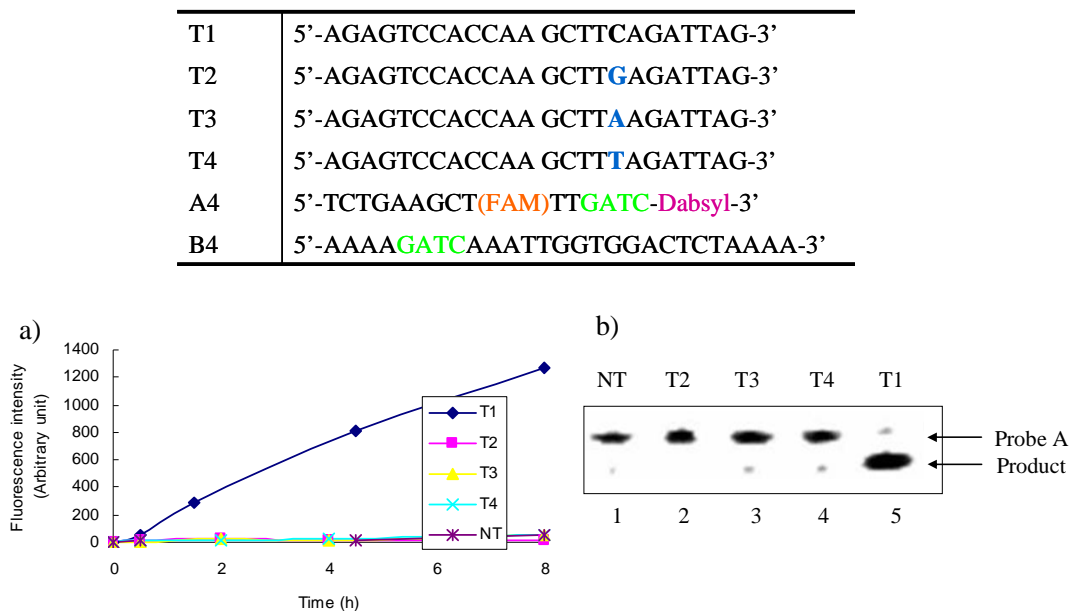


Figure 2.8 SNP detection. The table lists the probe and templates sequences used in the experiments. a) Fluorescence monitoring of the reactions. b) Gel analysis of the reactions. Reactions conditions: same as those for Figure 2.7.

2.9 Effect of target concentration on amplification

It is expected that the turnover (TO) of JP increases as the concentration of template decrease. 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 while the probe concentration were held constant (2 μM). The reactions were carried out at 30 $^{\circ}\text{C}$ for 5 hours. To ensure a meaningful comparison, we subtracted the background signals from the identical reactions lacking templates. When 0.25 equiv of matched template **T1** (500 nM) was used, the yield of the

reaction was 400% (corresponding to a turnover of 4, Figure 2.9). 0.05 and 0.01 equiv of T1 (100 and 20 nM) furnished product yields of 1323% (TO 13) and 3636% (TO 36), respectively (Figure 2.9). These results make us conclude that JP technology indeed has catalytic effect in DNA detection.

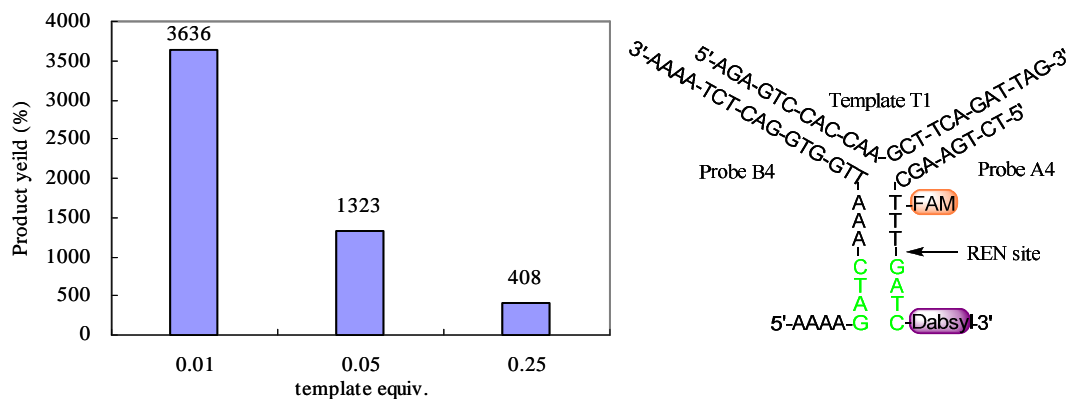


Figure 2.9 Catalytic efficiency of junction probes. Reaction time: 5 h. [Probes A] = [probe B] = 2 μ M. Product yield = $(x \cdot z)/y$ where x = fluorescence intensity of cleavage product, y = fluorescence intensity of cleavage product when template T1 = 2 μ M (a TO of 1 is assigned) and z = $[\text{probe A}]/[\text{T1}] \times 100\%$. Appropriate background subtractions (fluorescence intensity of reaction in the absence of a template) were made. Experiments were repeated 3 times and the error is less than 8%.

2.10 Picomolar DNA detection

As mentioned above, for our first-generation JP technology, a signal-to-noise ratio of 60 can be achieved. Because of the high signal-to-noise ratio of our technology, picomolar concentrations (femtomoles) of DNA could be detected (Figure 2.10).

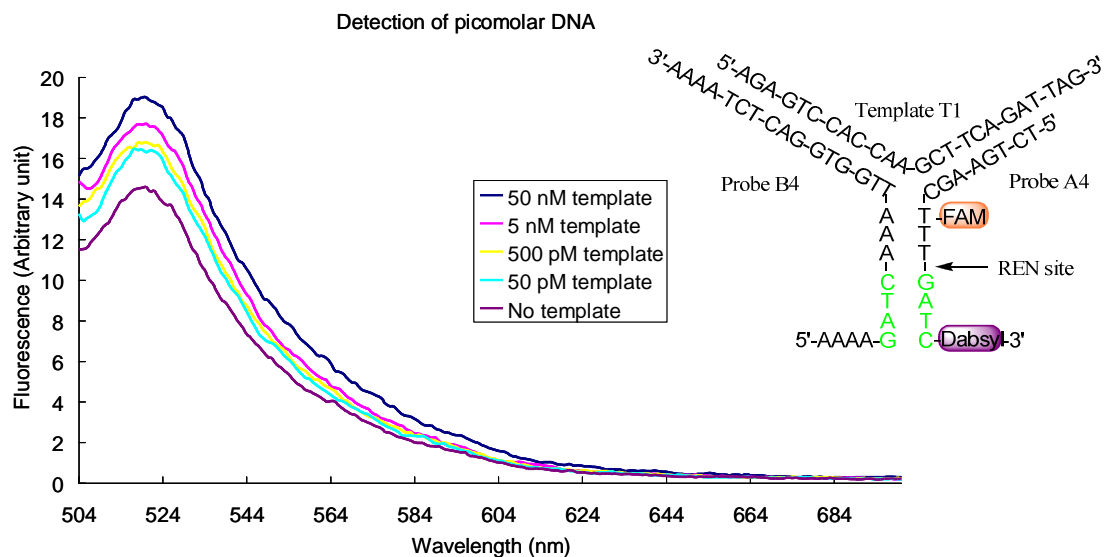


Figure 2.10 Picomolar DNA detection. Reaction conditions: [probe A] = [probe B] = 50 nM, [BfuCI] = 0.0267 U/ μ L, buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [DTT] = 1 mM, [BSA] = 100 μ g/mL. 30 °C, 9 h.

2.11 DNA thermal melting curves

The stability of the structures involved in JP technology is a major reason to explain the catalytic efficiency and selectivity. As mentioned before, the melting temperature of the duplex of probe A and probe B is lower than 15 °C (Figure 2.11a), while the melting temperatures of the matching template with probe A and probe B are higher than 30 °C (34 °C and 46 °C, respectively, see Figure 2.11b and 2.11c). The melting temperature of the junction structure is more complicated. Because DNA melting occurs step by step and domain after domain, the thermal melting of junction DNA structures such as complex **1** does not follow a simple two-state model and shows complex melting profiles (Figure 2.11d). Therefore, a single melting temperature T_m can not be assigned to junction structures **1**. The thermal melting curve of complex **1** (Figure 2.11d) shows two distinct apparent melting temperatures

(app. T_{m4} and T_{m5}) that are similar to the melting temperatures of probe A/template and probe B/template duplexes respectively (compare (b), (c) and (d) in Figure 2.11). We conclude that the presence of two distinct apparent melting temperatures in Figure 2.11d confirms that a junction structure is formed between probe A and B with the matched template.

In Figure 2.12, the melting curve of complex **1** clearly shows two distinct apparent melting temperatures (indicative of the formation of a junction structure) whereas that of complex **2** shows only one distinct melting temperature. Because the number of base pairing is reduced in complex **2**, it is not as stable as complex **1** and readily dissociates into binary and single components. That is the major rationale behind the catalytic cycle in JP technology.

For mismatch templates **T2-T4**, only one melting temperature (close in value to the T_m of probe B/template duplex) is observed (compare Figure 2.11b with Figure 2.13). This suggests that a single mismatch in the template prevents probe A from binding to the template to form a junction structure. Both fluorescence and gel-based assays (see Figure 2.8) further confirm that for mismatch templates **T2-T4**, junction structures are not formed because no cleavage products are observed when **T2-T4** are used as templates.

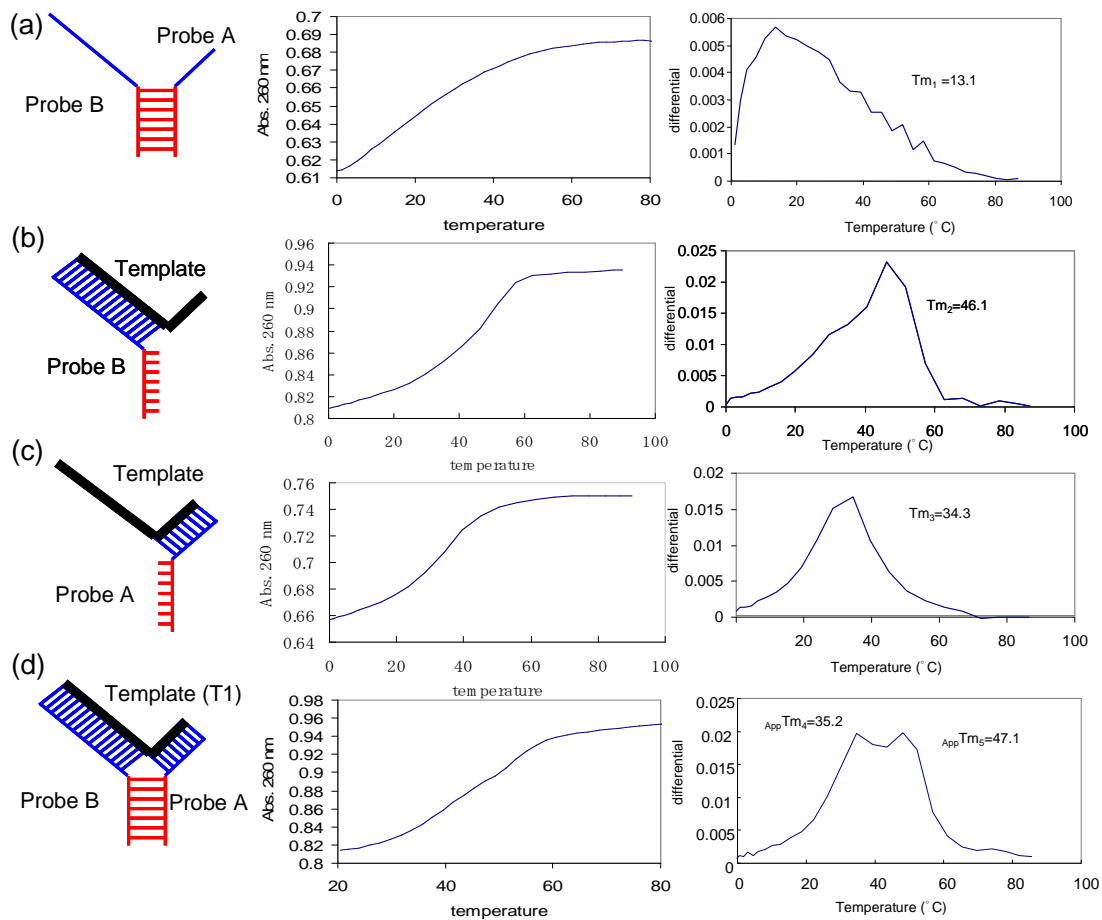


Figure 2.11 Melting curves of the duplexes and ternary complex. The left column shows the oligonucleotides components. The middle column shows UV absorbance change against the temperature change. The right column shows the melting temperatures (T_m) determined by the first derivative of absorbance with respect to $1/T$. Condition: [probe A] = [probe B] = [template] = 1 μ M, buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [DTT] = 1 mM. The mixture solution were heated to 85 $^{\circ}$ C, allowed to slowly cool at a rate of 1 $^{\circ}$ C/min to 4 $^{\circ}$ C.

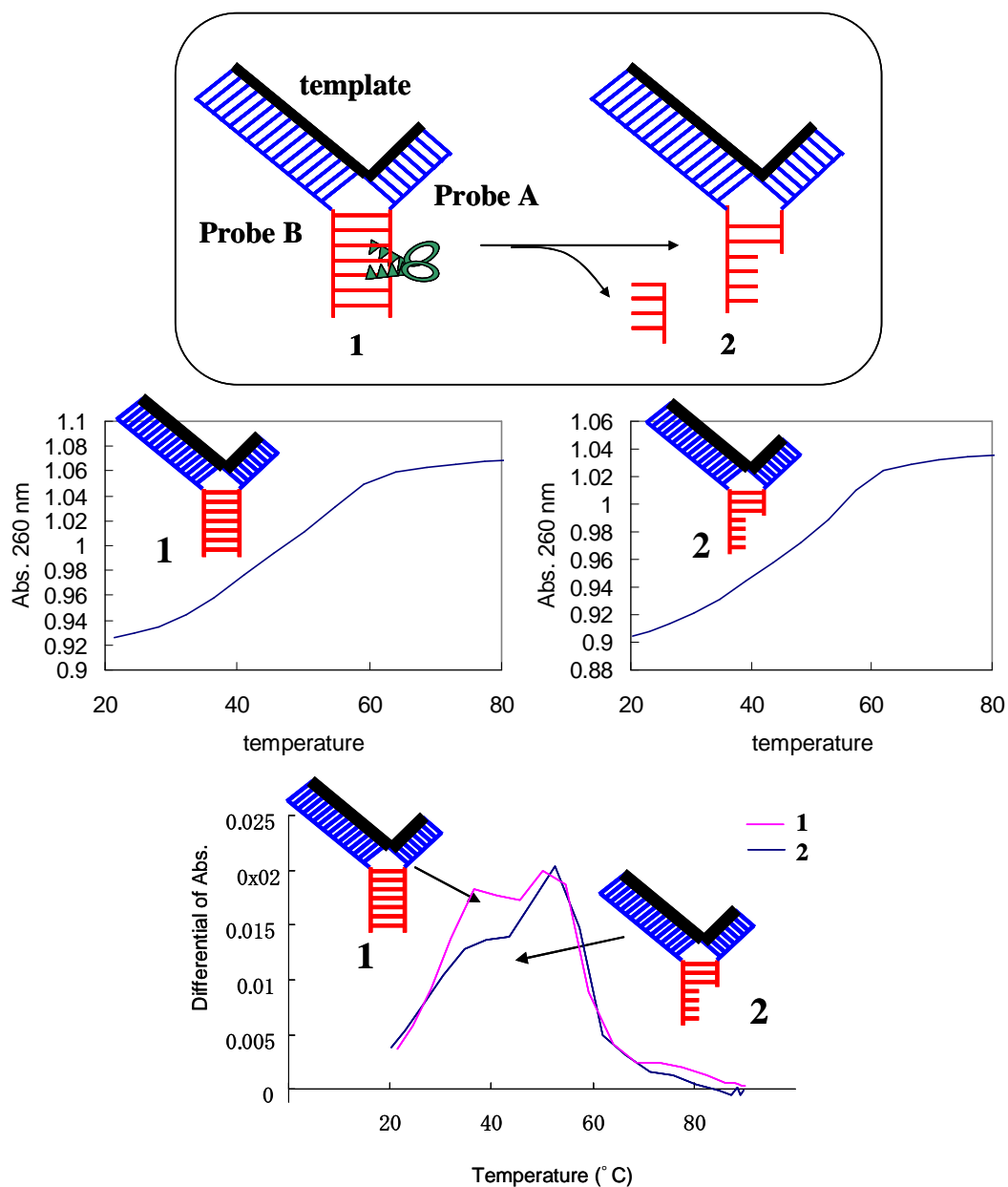


Figure 2.12 Melting curves of the ternary complexes **1** and **2**. The top row shows the transformation from complexes **1** to **2**. The middle row shows UV absorbance change against the temperature change. The right column shows the melting temperatures (T_m) determined by the first derivative of absorbance with respect to $1/T$. Condition: [probe A] = [probe B] = [template] = 1 μ M, buffer: [Tris-acetate] = 20 mM, [KOAc]

= 50 mM, $[Mg(OAc)_2] = 10$ mM, $[DTT] = 1$ mM. The mixture solution were heated to 85 °C, allowed to slowly cool at a rate of 1 °C/min to 4 °C.

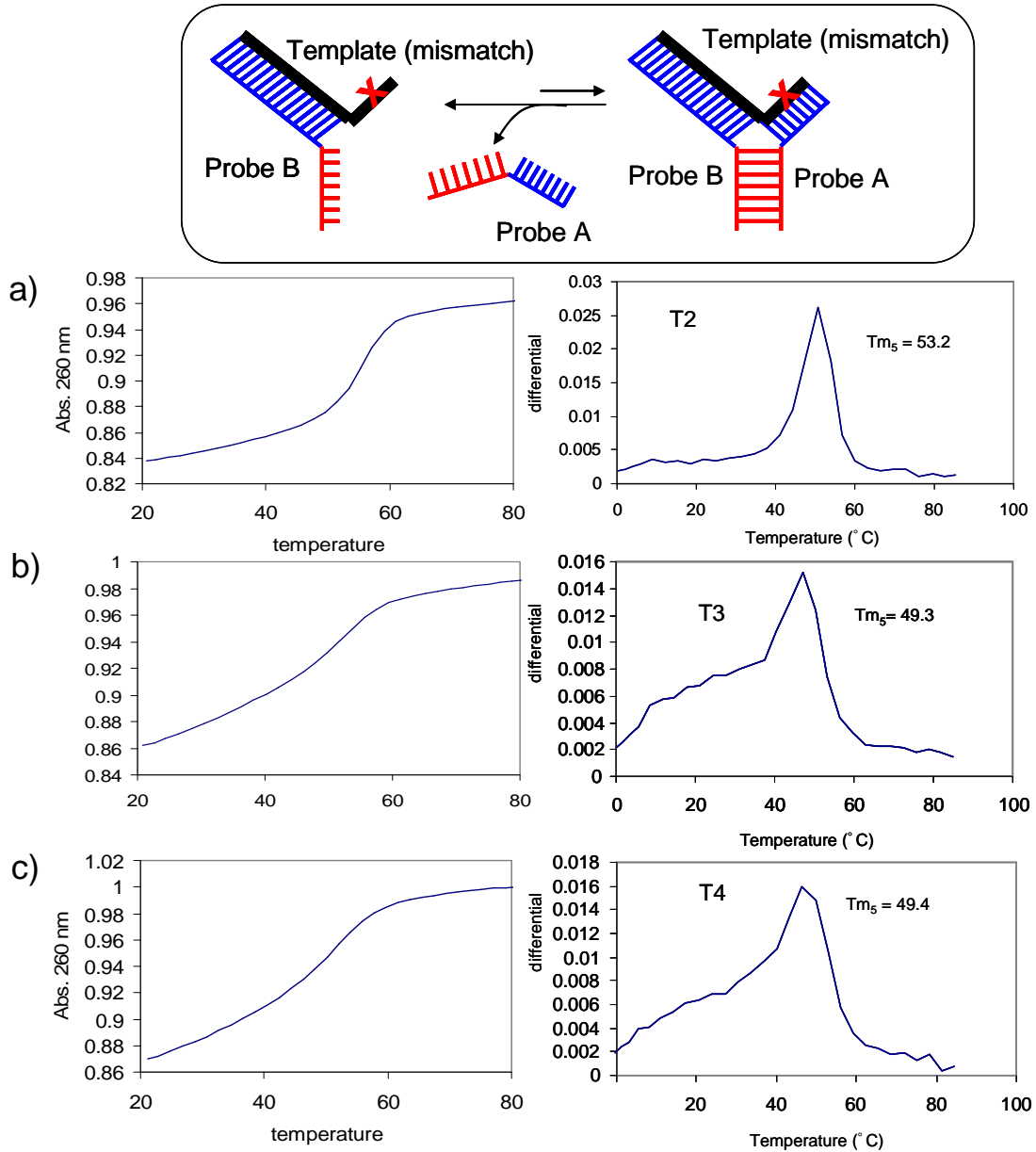


Figure 2.13 Melting curves of mismatched templates with the probes. The top row shows it is difficult for the mismatched template to form the junction structure. Below that, the left column shows UV absorbance change against the temperature change. The right column shows the melting temperatures (T_m) determined by the first

derivative of absorbance with respect to $1/T$. Condition: [probe A] = [probe B] = [template] = 1 μ M, buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [DTT] = 1 mM. The mixture solution were heated to 85 °C, allowed to slowly cool at a rate of 1 °C/min to 4 °C.

2.12 Alternative probe designs

As another effort to decrease the possibility of duplex **D2** formation (Figure 2.4), T-T base mismatch was introduced near the junction point by changing the sequence of probe B (Figure 2.14). T-T mismatches only destabilize a junction structure slightly,^{x3} while they should decrease the formation of **D2** considerably because the base pairing between probes A and B is reduced by 14% (from 7 to 6) in the alternative probe design (Figure 2.14). Note that noncognate sites (mispaird heteroduplex DNA, mutations within the recognition sequence) can be cleaved by numerous restriction enzymes,¹⁴⁰ but the influence of adjacent mismatches, to our best knowledge, has not been studied yet. For this approach, when the mismatch was one or two nucleotides away from the REase site, there was a significant change in the fluorescence intensity in the presence of target template **T1** but not in the absence of **T1**. However, when the mismatch was placed right next to the recognition site, the fluorescence intensity was at background level. Again, as the optimization of the FAM position disclosed previously, this result suggests that the enzyme BfuCI is sensitive to any change close to the recognition site. This design is particularly useful in reducing backgrounds, which is illustrated later.

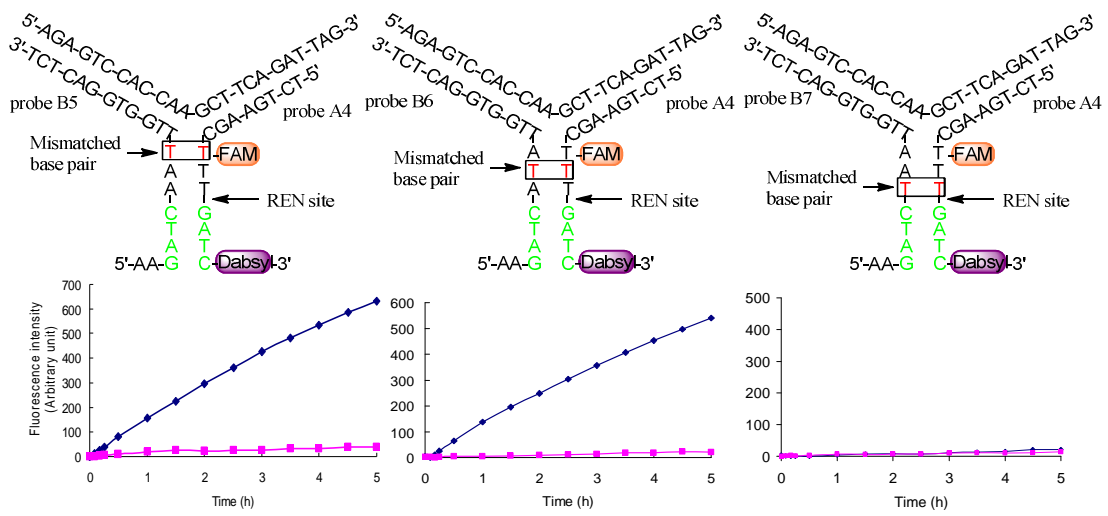


Figure 2.14 Alternative probe designs. Reaction conditions: same as those for Figure 2.7.

Chapter 3: Second generation of junction probe

In Chapter two, I described the first generation JP technology, which utilized the restriction endonuclease, BfuCI, to detect DNA templates which did not contain the cognate recognition sequence of BfuCI at isothermal conditions. Recently, we became more interested in detecting RNA. The detection of RNA¹⁴¹ is not as straightforward as that of DNA.¹⁴² In typical RNA detection schemes, the RNA is first copied into DNA before PCR is used to amplify the cDNA. Also the facile degradation of RNA samples by adventitious RNAses into smaller fragments during sample preparation makes the use of PCR-based methods to detect RNA not straightforward as PCR is better suited for longer transcripts. We aimed to detect RNA using restriction endonucleases, although restriction endonucleases do not generally recognize or modify RNA molecules. With the exception of few,¹⁴³ most technologies that utilize endonucleases to detect nucleic acids require the presence of specific sequences in the target analytes; therefore they can not be used to detect most genes. Direct adaptation of the first-generation JP to detect RNA was not as successful as the detection of DNA. This was partly because the first generation JP platform required very long assay times (several hours) in order to detect low concentrations of analytes and for biomolecules such as RNA which are not hydrolytically stable, such long assay times were not suitable. We therefore initiated a program to unravel salient design principles that would enable the development of a more efficient JP system.¹⁴⁴ The aims for the development of a second generation JP include:

1. Improving the time of the assay.

2. Detecting RNA target.
3. Detecting RNA in crude samples without extensive sample preparation.

3.1 Discovery of inhibition cycle of JP

In JP design, since the cleavage arm of the “Y” junction should be kept to a minimum to minimize the template independent hybridization, no base pairs are added at one side (the opposite end of the junction). However, for the successful cleavage of the cognate restriction endonuclease site, 5’-GATC-3’ in JP structures by BfuCI, it is imperative that there exist an oligonucleotide overhang after the cognate REase site (see Figure 3.1). A minimum of one nucleotide overhang was needed for effective cleavage by BfuCI. Overhangs of two or more nucleotides gave a faster cleavage reaction than a single nucleotide overhang. We therefore rationalized that if the oligonucleotide overhang in JP tripartite is cleaved by the enzyme during catalysis, then blunt JP structures will accumulate and inhibit the catalytic cycle (see Scheme 3.1, Inhibition cycle).

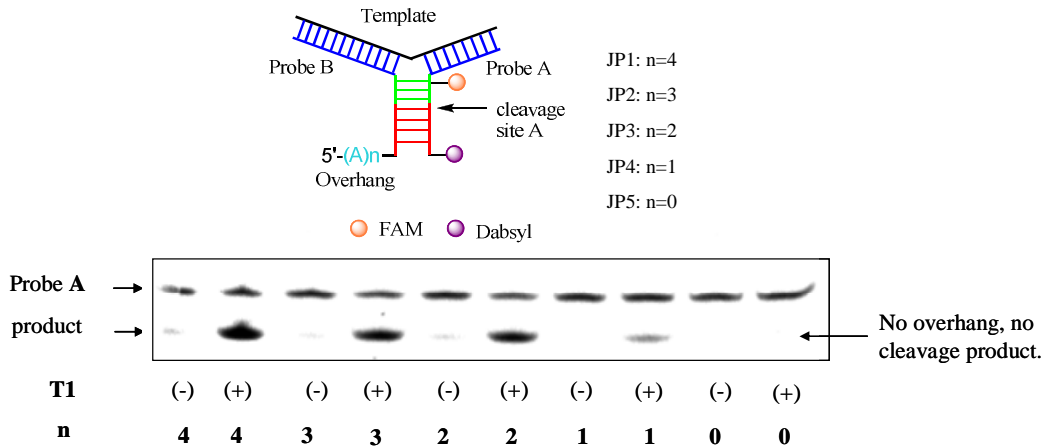
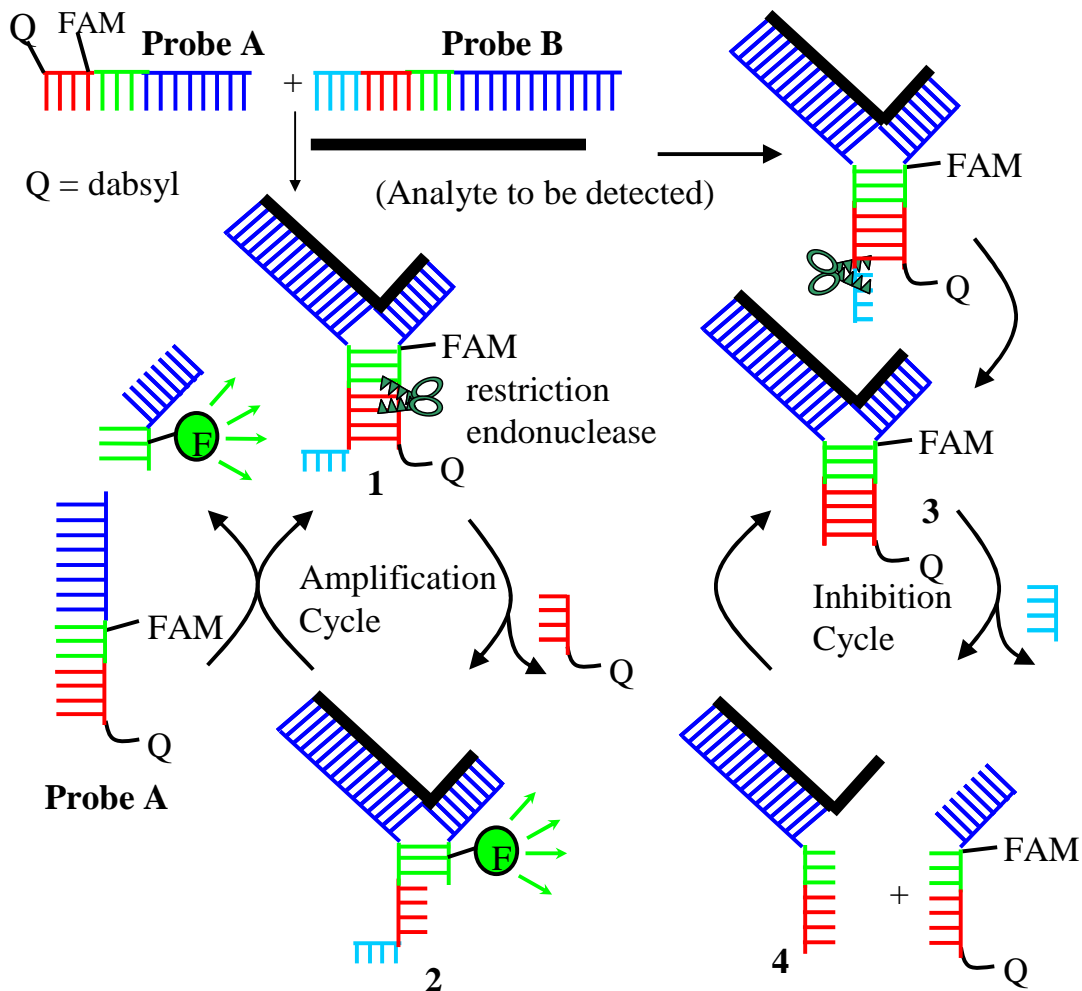


Figure 3.1 Overhang effect. Only JP structures that have 5’ overhang tail next to the cognate recognition sequence (colored red) are cleaved by the REase, BfuCI. Note

that there is no cleavage product for **JP5** ($n = 0$). Reaction conditions: same as those for Figure 2.5.



Scheme 3.1. Amplification and inhibition cycles of junction probe

The overhang in our JP platform is an absolute requirement for BfuCI cleavage. To determine if the overhang is also a requirement for other enzymes, the cleavage of JP structures with and without overhangs by two isoschizomers of BfuCI, MboI and DpnII, were investigated (Figure 3.2). From the gel (Figure 3.2a), it is clear that the overhang is vital for all three enzymes' cleavage since no product was observed when there was no single strand overhang beyond the REase recognition site. MboI was not

able to cleave DNA when the cognate site and/or nearby base was labeled with fluorophores. DpnII can handle labeled duplex DNA, however, the fluorescence data showed that it did not cleave the DNA in JP structure to an appreciable extent (Figure 3.2b).

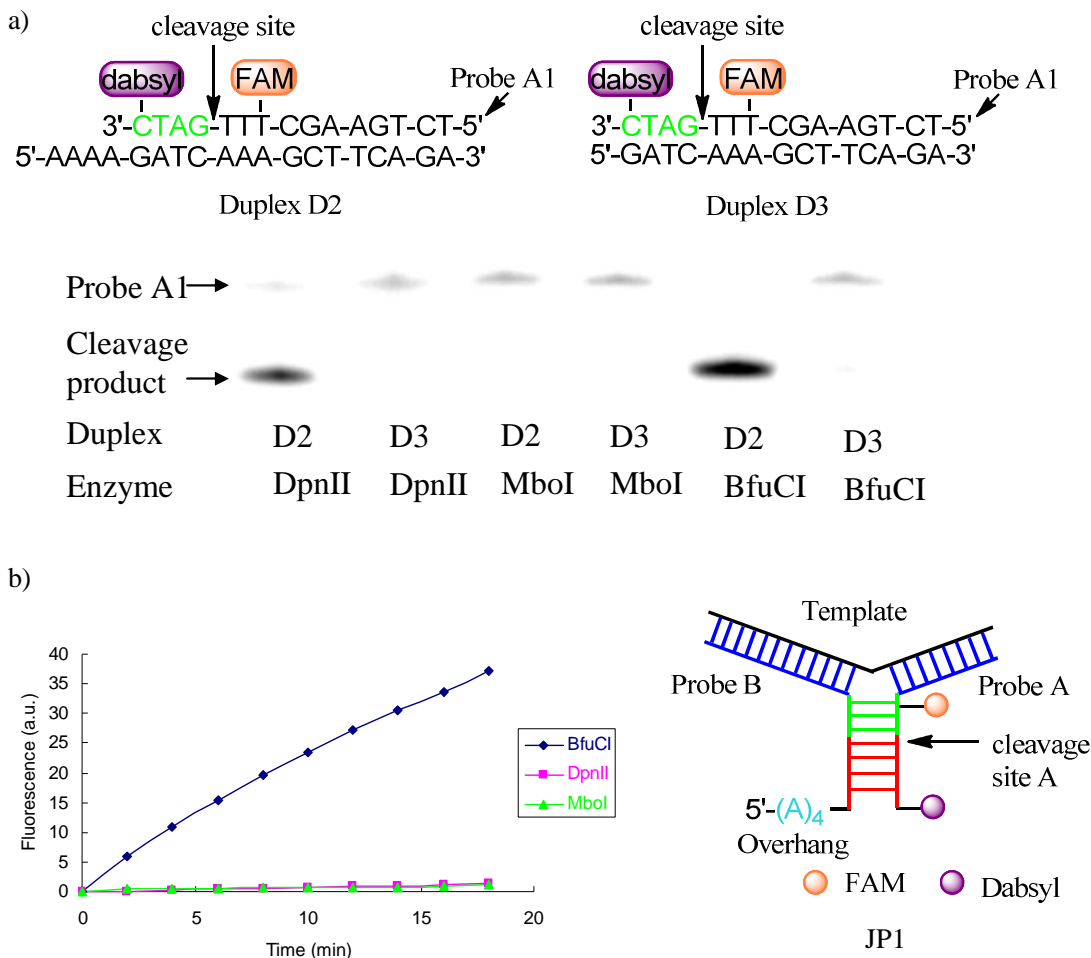


Figure 3.2 (a) Activity of DpnII, MboI (both isoschizomers of BfuCI) and BfuCI to fluorophore labeled DNA. Reaction condition: 2 μ M probes respectively, 0.067 U/ μ L enzymes respectively, 30 $^{\circ}$ C, 2 h. (b) Activity of the enzymes to the first generation of JP. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 522$ nm). Reaction condition:

200 nM probes A and B respectively, 1 nM template, 0.033 U/ μ L BfuCI; 0.0417 U/ μ L DpnII; 0.0417 U/ μ L MboI; 30 °C.

Incubation of JP probes A and B that had their respective 5'-ends labeled with fluorescein, matching template and the restriction endonuclease enzyme BfuCI revealed that both sites A and B of the double strands in **JP1** can be cleaved by the enzyme (see Figure 3.3). This result was initially of a surprise to us because it was expected that due to the lack of a duplex structure beyond site B in **JP1**, the rate of cleavage at site B would be significantly less than that at site A and hence blunt end and inhibitory products such as tripartite **3** (see Scheme 3.1) would not accumulate.

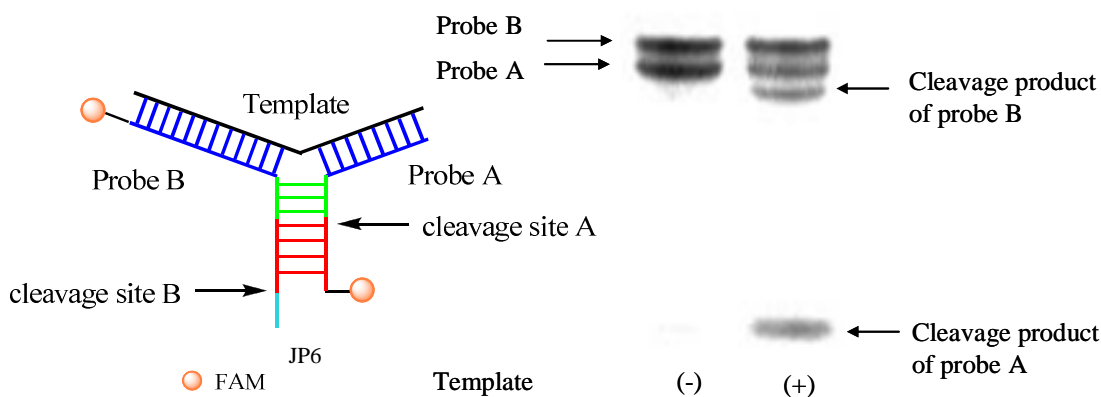


Figure 3.3 REase, BfuCI can cleave both sites A and B in **JP1**; although site B is adjacent to a single strand overhang. Reaction conditions: same as those for Figure 2.5.

3.2 Rationale behind the second generation JP

Single stranded DNA (ssDNA) cleavage of many REases has been reported.¹⁴⁵⁻¹⁵⁰ However, there is a controversy over the mechanism of ssDNA cleavage of REases. Some researchers have demonstrated that ssDNA fragments, which could not form a

double-stranded structure, were cleaved by REases.^{145, 146} Others have argued that the enzymes were acting on transiently formed double stranded DNA.¹⁴⁷⁻¹⁵⁰ In our JP case, the double-stranded recognition site of BfuCI probably facilitated the cleavage of ssDNA overhang, after the enzyme was bound to the double-stranded region.

Analyses of the X-ray crystal structures of five REases (BglI from *Bacillus subtilis*,¹⁵¹ EcoRI from *Escherichia coli*,¹⁵² FokI from *Flavobacterium okeanoikoites*,¹⁵³ HincII from *Haemophilus influenzae*¹⁵⁴ and NgoMIV from *Neisseria gonorrhoea*¹⁵⁵) whose structures are deposited in the protein data bank (PDB) revealed that the majority of these REases contact bases or phosphate moieties that lie a few nucleotides away from their cognate recognition sequences. For example, EcoRI makes contact with phosphate linkages that lay two nucleotides away from the cognate recognition sequence.⁷⁰ These interactions play an important role in the process of EcoRI-DNA complex formation.^{x157-x159} The crystal structure of the REase, BfuCI, has not been solved and its full biochemical characterization has not been undertaken, to the best of our knowledge. But it may also require the interactions with the base or phosphate outside of the recognition site for binding and/or cleavage.

To suppress the inhibition cycle, we need to prevent the cleavage of the overhang. Phosphate backbone modification of the REase recognition site appears to be an attractive choice since many studies have shown that most REases do not cleave REase cleavage sites that are modified with phosphorothioate,¹⁶⁰ phosphotriester,¹⁶¹ phosphoramidate,¹⁶² alkylphosphorothioate,¹⁶³ and alkylphosphonate¹⁶⁴ moieties. Connolly and co-workers examined a set of DNA dodecameric oligonucleotides containing the recognition sequence as cleavage substrates for EcoRV, in which

phosphorothioate linkages were introduced at certain positions, including the scissile linkage.¹⁶⁵ They found that when phosphorothioate modifications are placed outside the REase recognition site, for example GACp**GAT/ATCGTC**, **GAT/ATCGpTC** and **CGAT/ATCGTC** (p denotes phosphorothioate linkages and bold letters label the recognition sites), the rate of cleavage by EcoRV was similar to that of unmodified DNA. This was not surprising because analysis of the crystal structure of DNA, in complex with EcoRV revealed that the enzyme made little contact with the phosphate moieties outside the recognition sequence.¹⁶⁶ However, when oligonucleotides that contain phosphorothioate modifications in the recognition sequence were used (such as GACG**A**p**T/ATCGTC** and GACG**A**Tp**/ATCGTC**) the rates of cleavage by EcoRV were significantly less than that of the unmodified DNA. One can therefore turn a restriction endonuclease into a nicking endonuclease by modifying of the strands in the recognition site (especially at the site where cleavage occurs) with phosphorothioate modification.¹⁶⁷ Other backbone modifications such as methylphosphorothioate can also be used to block REase cleavage.¹⁶³

Many Type IIP restriction enzymes are dimers of identical subunits that interact symmetrically with their recognition sequences. In a hydrolysis reaction, a dimer binds to one duplex recognition sequence, and one active site in the dimer cleaves one strand of the DNA, while the second active site cleaves the symmetrically equivalent position in the other strand of the DNA. However, several studies have reported restriction endonucleases that differ from the orthodox in that they require two copies of a recognition sequence to cleave the DNA.¹⁶⁸⁻¹⁷⁵ These restriction endonucleases, which require two recognition sites for optimum activity, belong to different subtypes

of Type II restriction endonucleases, namely Types IIE, IIF and IIS (Figure 3.4), *vide infra*. Note that current nomenclature of classifying the Type II REases does not avoid overlap between different subtypes. For example, NgoMIV (5'...G/CCGGC...3') is both a type IIP and IIF enzyme. Type IIE REases are homodimeric proteins that simultaneously bind two recognition sites to cleave the DNA within or near their recognition sites (Figure 3.4). One of the best understood Type IIE REases, NaeI,¹⁶⁸ cleaves supercoiled plasmids with two recognition sites faster than plasmids with a single site.¹⁶⁹ It also cleaves catenanes that consist of two plasmid rings with a single recognition site in each ring faster than plasmids with a single site.¹⁶⁹ Type IIF and IIE REases are different in that Type IIE enzymes are dimeric but Type IIF enzymes are homotetrameric. In addition, Type IIE REases predominantly release the full-length linear DNA that results from cleavage at one of the two sites, whereas Type IIF REases convert a two-site plasmid directly to the products that result from concerted cleavage at both sites (Figure 3.4), which are cut in a concerted reaction (for example, SfiI, Cfr10I, NgoMIV).¹⁷⁰⁻¹⁷² Type IIS REases recognize asymmetric DNA sequences and cleave at a defined distance downstream of the binding site.¹⁷³ Examples of Type IIS enzymes, which cleave two-site substrates faster than one-site, include BsgI, BpmI, Acc36I, FokI and BspMI.^{174, 175}

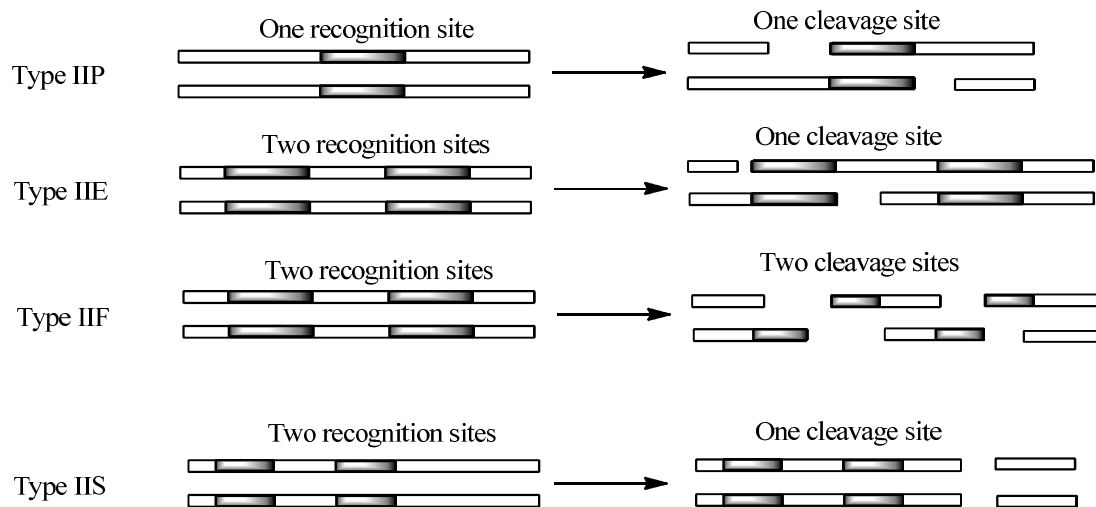


Figure 3.4 Illustrations of the recognition sites and cleavage styles of Type IIP, Type IIE, Type IIF and Type IIS restriction endonucleases.

3.3 Effect of modifications at site B of JP

Having established that cleavage at site B in **JP6** was indeed occurring, we proceeded to investigate if by modifying site B with nuclease resistant moieties, the accumulation of blunt-end tripartites such as **3** would be reduced, invariably leading to an increase in JP amplification rate.

Pleasingly, the modification of site B of JP with phosphoromonothioate (**JP7**) or phosphorodithioate (**JP8**) led to seven and three fold enhancements of the fluorescent intensity respectively over the original JP structure (**JP1**) whereby site B contained the native phosphate linkage (Figure 3.5a). Although the replacement of the exocyclic oxygen in the phosphate linkage at site B with either an acetate (**JP9**) or a methoxy group (**JP10**) completely prevented site B cleavage by BfuCI, the rate of **JP9/10** cleavage by BfuCI did not differ from that of the native phosphate moiety (**JP1**) (see Figure 3.5a). Several hypotheses can be formulated to account for the lack of rate

enhancement for the REase cleavage of **JP9/10**, compared to **JP1**. We favor this hypothesis; non-steric mimics of the exocyclic oxygen of the phosphate moiety inhibit either endonuclease/DNA complex formation or the DNA cleavage step. In line with this hypothesis, phosphoromonothioate (**JP7**) which is a better isostere of phosphate than phosphorodithioate (**JP8**) was cleaved by BfuCI at a faster rate than **JP8**, see Figure 3.5a.

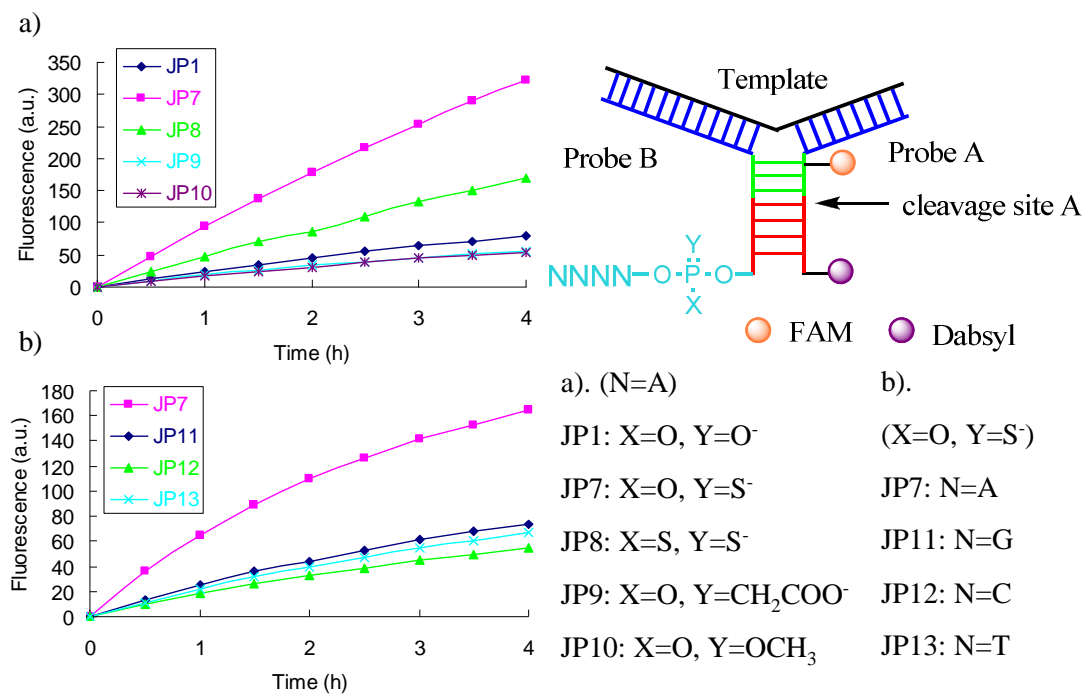


Figure 3.5 Effect of modification at site B. a). The cleavage rates of junction probes with different modifications at site B are significantly different. Phosphorothioate modification at site B (**JP7**) lead to the fastest cleavage rate. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$). [Probes A] = [probe B] = $2 \mu\text{M}$, [template] = $0.2 \mu\text{M}$, [BfuCI] = $0.033 \text{ U}/\mu\text{L}$, assay temperature = $30 \text{ }^\circ\text{C}$. b). In Figure b, all probes are modified at site B with phosphorothioate. The sequence contents of the probe B 5'-overhangs are however different. Fluorescence data is normalized.

[Probes A] = [probe B] = 1 μ M, [template] = 0.5 μ M, [BfuCI] = 0.033 U/ μ L, assay temperature = 30 $^{\circ}$ C.

Therefore, it was expected that the sequence content of the single strand overhang of JP tripartite would influence the rate of REase cleavage of JP structures. In line with this expectation, JP tripartite with four adenine overhang at the 5'-end of probe B (**JP7**) was cleaved three times faster than those containing four guanines (**JP11**), cytosines (**JP12**) or thymines (**JP13**), see Figure 3.5b.

3.4 The kinetics of BfuCI on modified substrate

To understand the influence of DNA modifications on enzymatic cleavage, we investigated the kinetics of BfuCI cleavage of several modified substrates. A stem-loop structure, modified with a fluorophore and a quencher was used as a model substrate to investigate how various phosphate modifications next to the BfuCI recognition site (at the site of overhang, which is also a potential cleavage site) could influence the rate of cleavage at the unmodified site (Figure 3.6). The initial fluorescence change of the reaction (within 2 min) was recorded and converted to reaction velocity, using a calibration curve (Figure 3.6a). From the linear plot of the Michaelis–Menten equation, V_{max} and Km values of 128 nM/min and 446 nM were obtained for the phosphoromonothioate substrate, 48 nM/min and 1054 nM for the methyl phosphotriester substrate (Figure 3.6c and d). However, the data of natural phosphodiester substrate did not fit the linear plot (Figure 3.6b).

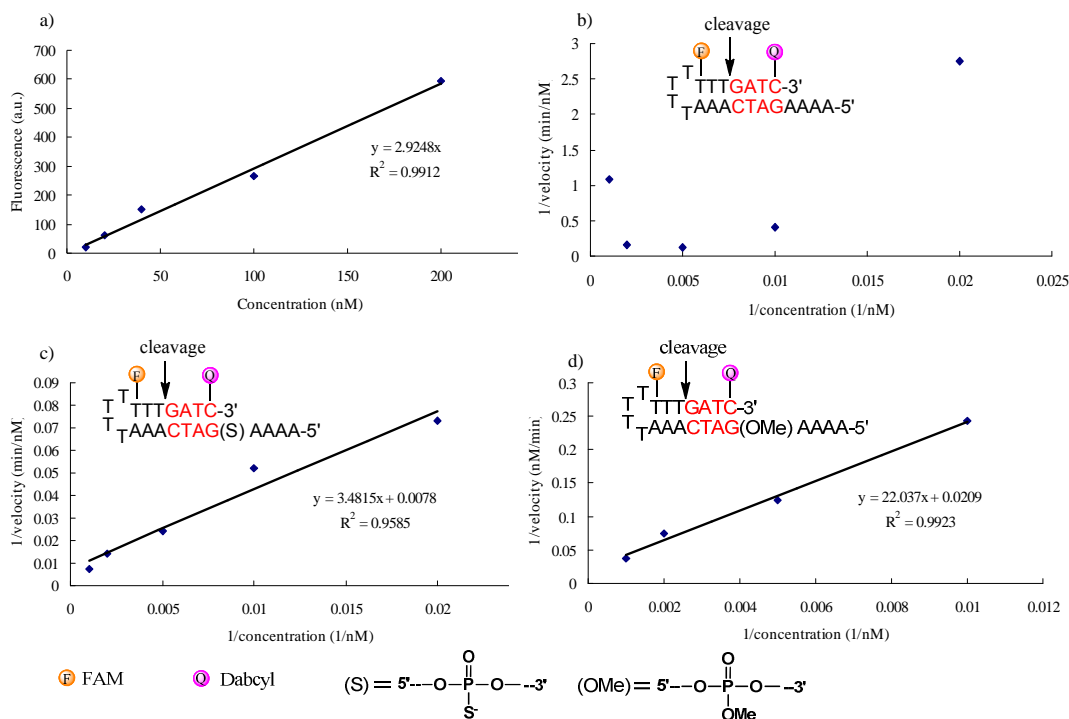


Figure 3.6 Kinetics studies of BfuCI cleavage of modified substrates. (a) Fluorescence-DNA calibration curve. A plot of fluorescence of fluorescein labeled DNA against the concentration determined from UV measurement. (b)-(d): double-reciprocal plot of kinetic data of BfuCI with natural substrate (b), phosphoromonothioate substrate (c) and methyl phosphotriester substrate (d).

3.5 Effect of architectures of the overhang

3.5.1 Duplex effect

The discovery that the sequences that lie adjacent to the cleavage site in JP tripartites significantly influence the cleavage rate of JP structures prompted us to investigate how other architectures, such as duplex overhangs, would influence the REase cleavage rate of JP tripartites. Consequently **JP14-22** were designed to investigate if the presence of a duplex overhang at the 5'-end of probe B would be

beneficial to junction probe catalysis. In **JP14**, **17** and **18**, the duplex overhang does not lie immediately after the REase recognition site but is rather appended to a four-nucleotide single-stranded overhang. On the contrary, the 5'-duplex overhangs in **JP16**, **19-20** lie immediately next to the REase recognition site. It must also be noted that whereas **JP14-16** contain no mismatched base pairs near the endonuclease cleavage site (see Figure 3.7), for the design of **JP17-20**, mismatch base pair sites are introduced at a region that is close to the junction structure (Figure 3.8). Two interesting observations result from junction probe designs **JP14-16** and **JP7**. Firstly, the presence of a duplex overhang at the 5'-end of probe B in JP tripartite enhances the rate of JP cleavage by the REase, BfuCI. For example, the average rate of cleavage of **JP16** (which contains a duplex overhang immediately preceding the REase duplex recognition site) in the first 5 min is about 40 times faster than that of **JP7** (which contains a single strand overhang immediately preceding the REase duplex recognition site). Also, the cleavage reaction of **JP16** is almost complete after 25 min whereas that of **JP7** is not complete after 4 h (240 min, see Figure 3.5b). Secondly, the signal-to-noise ratio (S/N; i.e. fluorescent signal generated in the presence of a matching template divided by that generated in the absence of a template) of **JP16** is 1.3 after 25 min (when the cleavage reaction is complete for **JP16**) whereas that of **JP7** is 22. Therefore, although the introduction of a duplex overhang next to the REase recognition site significantly accelerated the cleavage reaction, the deplorably low S/N prompted us to investigate strategies that would lower the signal-to-noise ratio.

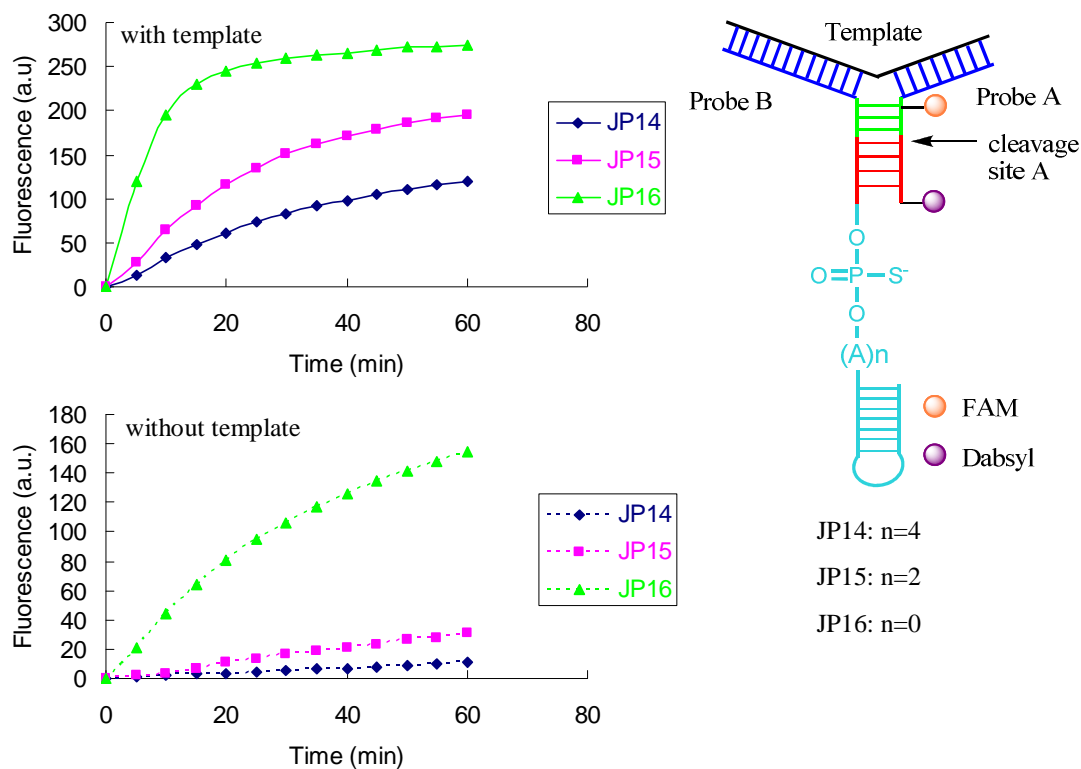


Figure 3.7 JP structures with a duplex overhang at 5'-end of probe B are cleaved faster than those with single strand overhangs. Fluorescence data: $\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$. Reaction conditions were the same as those for Figure 3.5b.

In order to retain the beneficial effect of placing a duplex overhang next to the cleavage site of BfuCI but reduce the unacceptable S/N, we explored the effect of reducing the base-pair complementarity between probes A and B. As reported in our previous work, for the effective cleavage of JP tripartite structures, it is important to have a “buffer zone” (ideally three nucleotides or greater) between the REase cleavage site and the junction site (Figure 2.5). Therefore, the only means that was available to reduce the base-pair complementarity between probes A and B (and hence reduce background noise) and still satisfy the buffer zone requirement was to introduce a mismatch base-pair in the buffer zone as mentioned earlier (Figure 2.14).

One mismatch was introduced in the buffer region near the endonuclease cleavage site in **JP17-20**. No mismatch base pairs were allowed immediately next to the cleavage site because we had earlier observed that the introduction of that completely abolished the cleavage reaction (see Figure 2.14). Pleasingly, the introduction of a single mismatch base-pair in the buffer region of junction probes (see Figure 3.8) resulted in dramatic changes in S/N. For example **JP17/18** and **JP14** have similar structures, except that both **JP17** and **18** have single mismatch base-pairs in the buffer zone whereas **JP14** does not contain any single mismatch base-pairs in the buffer zone, but the S/N of **JP17** and **18** after 25 min are 13.5 and 9 respectively whereas that for **JP10** is 4.7. Similarly, S/N for **JP20** is 10 whereas that for **JP16** is significantly lower (1.3); although the two probe designs are similar, except for the presence of a single mismatched base-pair in the buffer zone of **JP20**.

In order to study the effect of the duplex overhang to the cleavage rate, we ranged the base pair numbers from 8 to 32 based on **JP20** structure since it has both fast cleavage rate and low S/N value. The result showed that different base-pairing number of the 5'-duplex overhang gave similar REase cleavage rate (Figure 3.9).

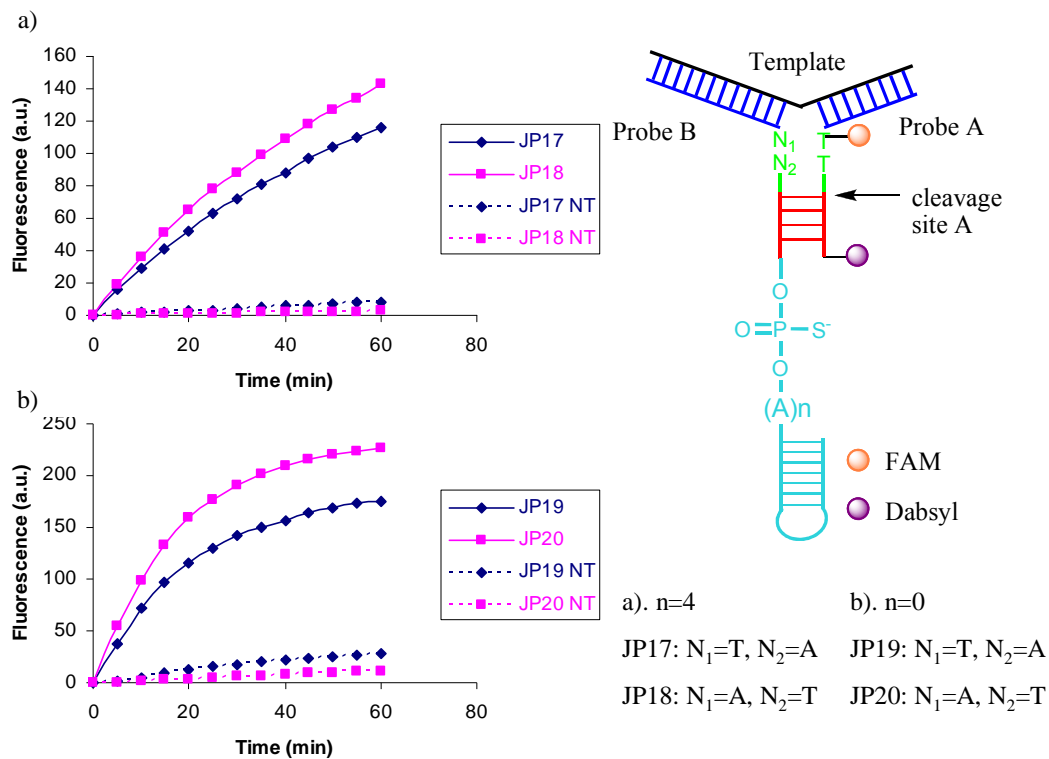


Figure 3.8 The reduction of the number of base-pairing between probes A and B result in lower background noise. Fluorescence data: $\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$. Reaction conditions are the same as those for Figure 3.5b; NT = no template was added (i.e. background noise)

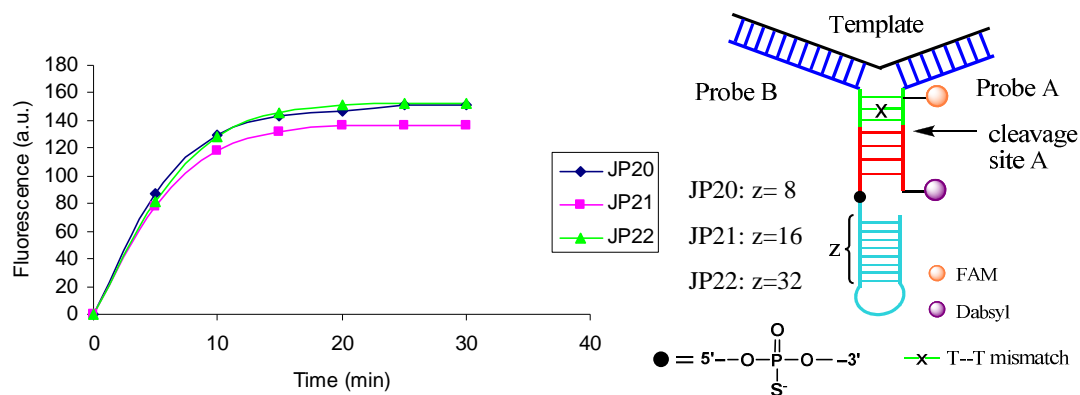


Figure 3.9 The length of 5'-end duplex overhang of probe B is inconsequential for the rate of JP cleavage by BfuCI. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$). Reaction conditions are the same as those for Figure 3.5b.

3.5.2 Multiplicity of REase recognition sites

REases can form monomers, dimers, or tetramers in solution. They can also bind a single recognition site or two DNA sites simultaneously.¹⁷⁶ It has been demonstrated that a growing number of type II restriction endonucleases such as EcoRII,¹⁷⁷ NgoMIV,¹⁷⁸ SgrA1,¹⁷⁹ SfiI,¹⁸⁰ Cfr101¹⁸¹ etc. require a second cognate recognition site for allosteric activation. We therefore designed junction probe structures **JP23-27** to contain additional recognition sites in the duplex overhang at the 5'-end of probe B to investigate if the presence of an additional cognate binding site would allosterically activate the cleavage of JP structures by BfuCI. As probe B is not cleaved during JP catalysis, it would be advantageous if probe B remains affixed to the target analyte during the catalytic process. Therefore, it is desirable that the second REase cognate recognition site that is placed on the 5'-duplex overhang of probe B contains nuclease resistance sites so that the duplex moiety remains intact during catalysis. Consequently, in **JP23-27**, the second recognition site on the duplex overhang was

modified with phosphoromonothioate at the two cleavage sites. In Figure 3.10, the results showed that **JP23** was cleaved twice as fast as **JP20**. Although a factor of two is a modest enhancement, it indicates that BfuCI might belong to the growing class of type II REases that are allosterically activated by a second recognition site. The addition of a third recognition site in the duplex overhang region of probe B (see **JP24**) did not lead to any further enhancement of cleavage rate. The architecture of the 5'-overhang is also important for JP cleavage. Overhangs that contain additional recognition sites but that differ in structure from the canonical duplex overhang in **JP23** led to a dramatic decrease in JP cleavage rate; indicating that steric encumbrance is not tolerated by the enzyme (see Figure 3.10, **JP25-27**).

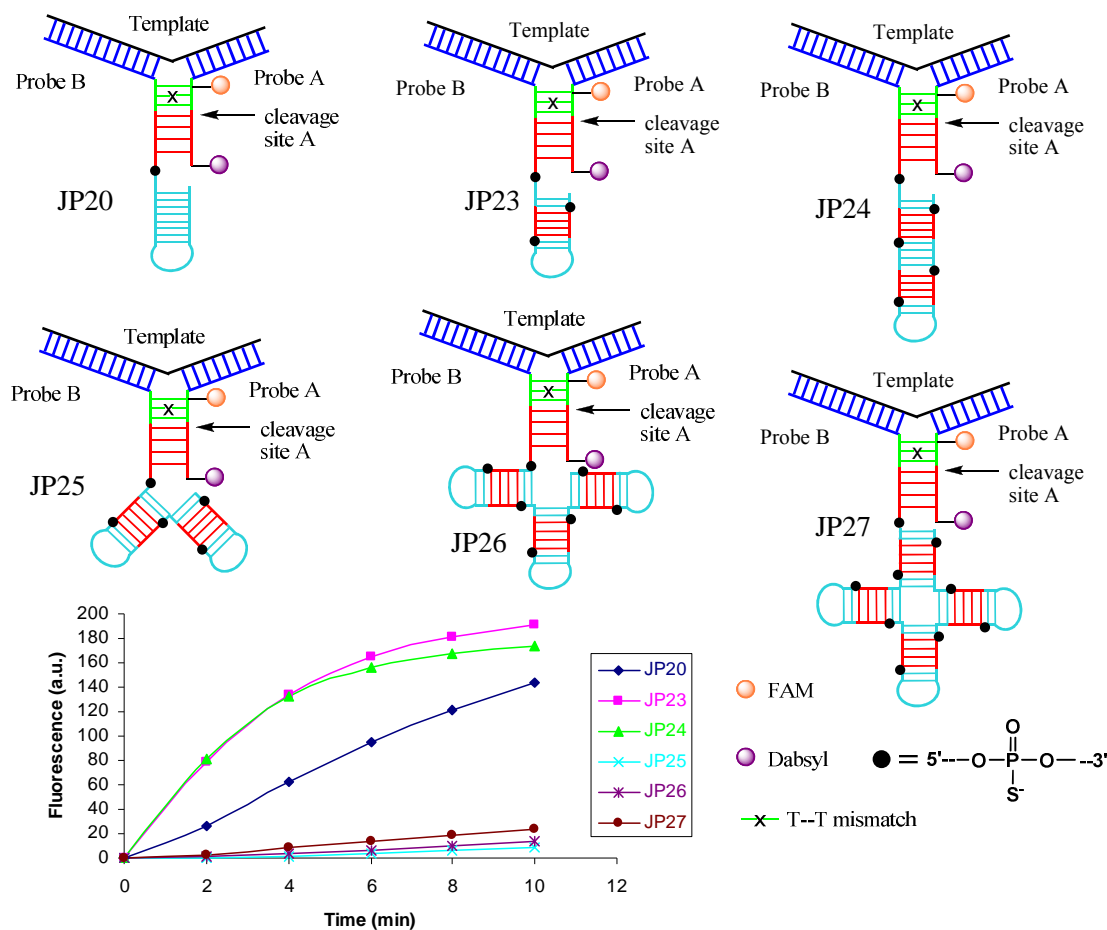


Figure 3.10 The presence of REase recognition site in the duplex overhang at the 5'-end of probe B result in enhanced cleavage of junction probes by BfuCI. The second recognition site in the duplex overhang is made nuclease-resistant via phosphoromonothioate modification. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$). Reaction conditions were the same as those for Figure 3.5b.

For the proposed catalytic cycle of the junction probe technology, the two fragments of probe A that result from REase cleavage fall off tripartite structure **2** and is replaced by a new uncleaved probe A (see Scheme 3.1). It therefore follows that factors that influence either the on-rate of probe A or the off-rate of the cleavage product of probe A will affect the rate of JP amplification. We therefore investigated

the effect of probe A-template base-pairing number (i.e. the number of base pairs between probe A and the template; designated as x in Figure 3.11) on JP cleavage rate. Our expectation was that the JP cleavage rate would increase as x increases (due to increase in on-rate) up to a particular x value and begin to decrease as the value of x increases further (because the off-rate for the cleaved tripartite **2**, see Scheme 3.1, would decrease as x increases). In line with this expectation, the rate of JP cleavage by BfuCI is as follows; $x = 7 < 8 < 9 < 10 > 11 > 12$ (Figure 3.11). Similarly, lower base-pairing number between probe B and template e.g. **JP36**; $y = 8$ (which implies lower stability of probe B/template duplex) leads to slower cleavage rate of JP tripartite by BfuCI (Figure 3.12).

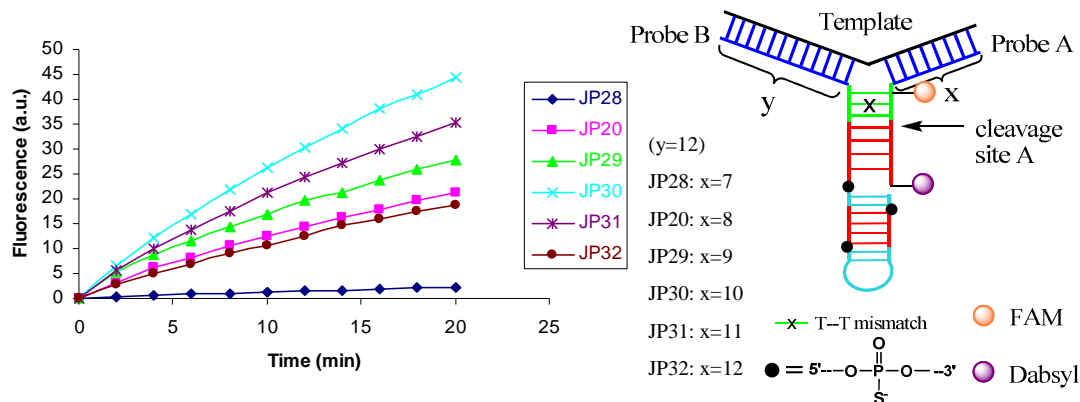


Figure 3.11 The base-pairing numbers between template and probes A (designated as x) influence the cleavage of JP tripartite by BfuCI. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$, Ex slit = Em slit = 10 nm, PMT = 600 V). Reaction conditions: [Probes A] = [probe B] = 200 nM, [template] = 1 nM, [BfuCI] = 0.1 U/ μL , assay temperature = 35 °C.

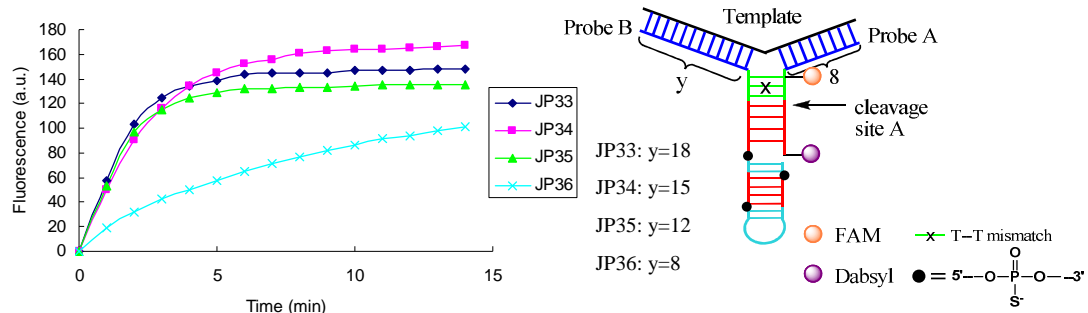


Figure 3.12 The base-pairing numbers between template and probes B (designated as y) also influence the cleavage of JP tripartite by BfuCI. Reaction conditions were the same as those for Figure 3.5b.

3.6 Effect of concentration of BfuCI for cleavage assay

The restriction endonuclease, BfuCI, plays an important role in JP technology. Thus the effect of its concentration was studied as follows. From the results, we can see that in a certain range, the concentration of the enzyme has not much influence on the cleavage rate (see Figure 3.13).

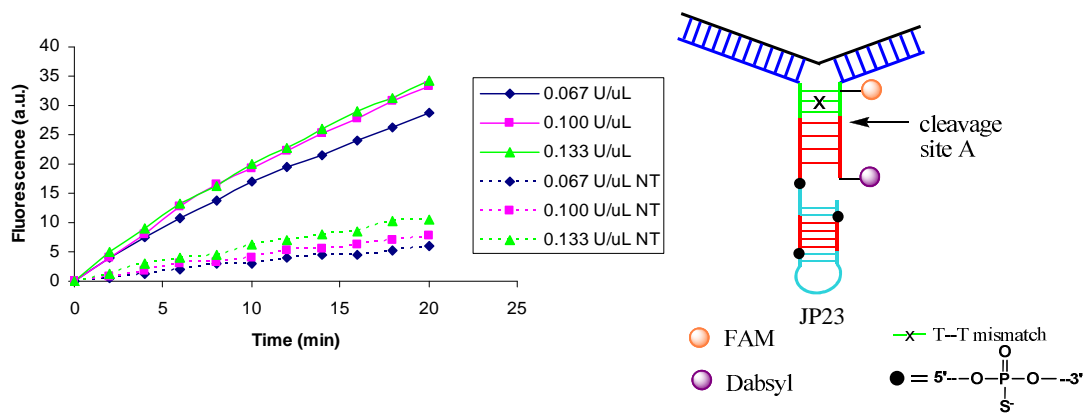


Figure 3.13 The concentration of the enzyme has not much influence on the cleavage rate. Reaction condition: [probe A] = [probe B] = 200 nM, [template] = 1 nM, assay temperature = 30 °C. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 522 \text{ nm}$, Ex slit = Em slit = 10 nM, PMT = 600 V).

3.7 Stability of the hairpin overhang

As discussed in the last chapter, the profile of junction is complicated. While in our second generation of JP, a hairpin overhang is introduced at the 5' end of probe B. To ensure this hairpin is stable at the assay temperature, we investigated the melting point of the hairpin overhang. The observation showed that the hairpin has a high melting point (75 °C, see Figure 3.14). Therefore we believe the hairpin was the dominant form at the assay temperature (30 °C).

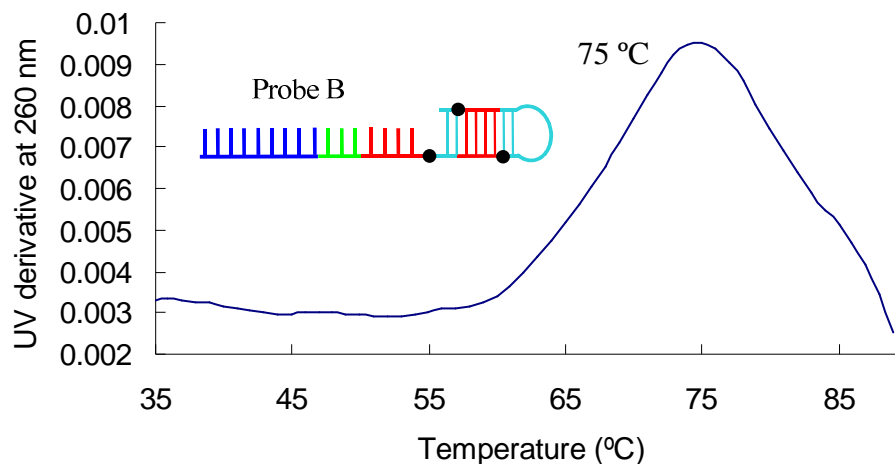


Figure 3.14 Melting curve of probe B with a hairpin overhang. Condition: [probe B] = 3 μ M, 20 mM Tris-acetate, 50 mM KOAc, 10 mM Mg(OAc)₂, 1 mM DTT, and pH 7.9.

3.8 Comparison with molecular beacon (MB) and the first generation JP

The second generation JP was compared with molecular beacon and the first generation JP. The results showed that the second generation JP is more sensitive and

achieved two orders of magnitude higher signal than MB, and one order of magnitude higher signal than the first generation (Figure 3.15).

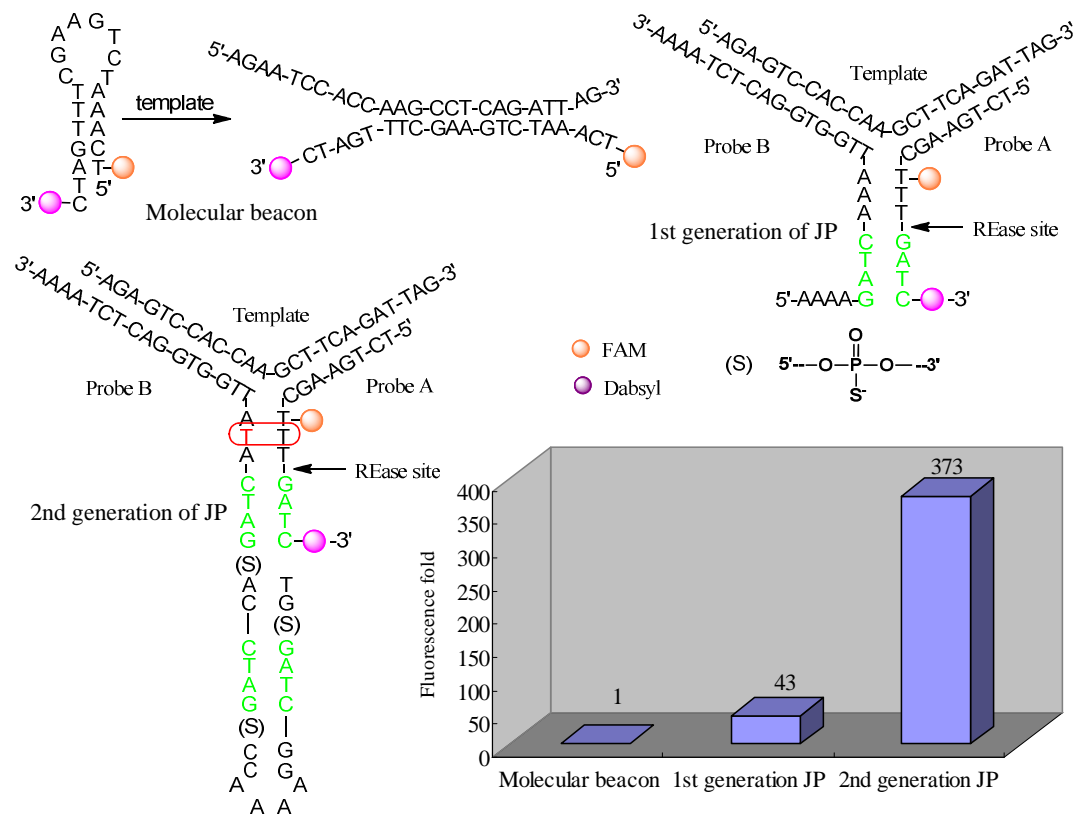


Figure 3.15 Comparison of molecular beacon (MB), first generation and second generation of junction probe. Reaction conditions: [probe A] = [probe B] = 2 μ M, [template] = 5 nM, [BfuCI] = 0.067 U/ μ L, buffer: [Tris-acetate] = 20 mM, [KOAc] = 50 mM, [Mg(OAc)₂] = 10 mM, [DTT] = 1 mM, [BSA] = 100 μ g/mL. 30 $^{\circ}$ C, 3 h. Fluorescence parameters: λ_{ex} = 494 nm, λ_{em} = 518 nm. 30 $^{\circ}$ C, 3 h. Experiments were repeated 3 times and the error is less than 5%.

3.9 Y-shaped complex formed on double helical DNA

In the aforementioned experiments, the template of JP detection was single stranded DNA. We would like to expand the template scope to double stranded DNA

sequences. Dervan and his co-workers have reported a Y-shaped complex formed on double helical DNA.¹⁸² In this type of structure containing a triplex, the third strand forms hydrogen bond interactions with the acceptor and donor groups in the DNA major groove. The hydrogen bond interactions between the third strand and the double helices are different from the Watson-Crick hydrogen bonds and referred to as Hoogsteen hydrogen bonds.¹⁸³ One strand of the double helices contains only purines [poly(AG)] and the other contains only pyrimidines [poly(TC)]. The two oligonucleotide probes are comprised of three domains: duplex recognition domain, linker, and dimerization domain (Figure 3.16a). The recognition domains specifically recognize segments of the template and form a triple helix through Hoogsteen hydrogen bonds (TAT and GCG triplets);^{184, 185} the linker gives flexibility at the junction, and the complementary dimerization domains of the two oligonucleotides can form duplex through Watson-Crick hydrogen bonds. One probe was modified at 5'-termini with thymidine-EDTA (*T), which could cleave the complex¹⁸⁶ (Figure 3.16b). They found that the modified probe alone did not bind to the double stranded template, but only with the assistance of the other probe and the formation of a cleavage product indicated the formation of the Y-shaped structure. The stability of the complex could be adjusted by changing the length and the content of the dimerization domain, while it could also be promoted by small ligand binding (Figure 3.16c).¹⁸⁷ When echinomycin binding site was incorporated in the deimerization domain, the small molecule echinomycin enhanced the probe-template binding by a factor of 3.2.

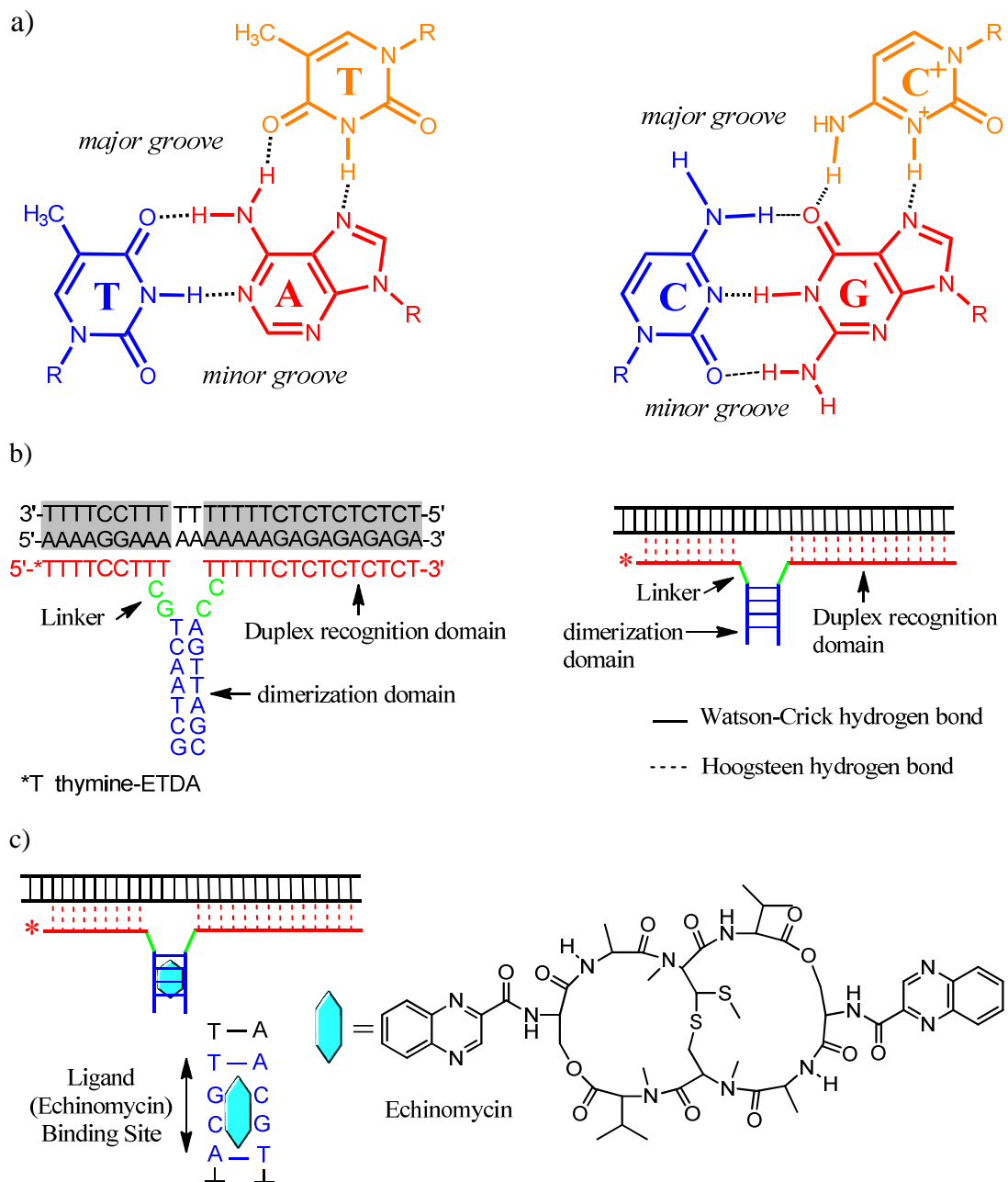


Figure 3.16 A Y-shaped complex composed of two triplex forming oligonucleotides. a) TA·T and CG·C⁺ triplet motifs, whereas the purine is in red, the pyrimidine is in blue, and the third base is in yellow. b) A Y-shaped complex formed on double helical DNA. c) A Y-shaped complex formed on double helical DNA stabilized by small molecule echinomycin.

3.10 Application of the second generation JP

3.10.1 DNA detection

To demonstrate the versatility of the JP platform for the detection of nucleic acids, we proceeded to investigate if this new generation platform can detect other clinically relevant nucleic acid sequences. The new generation junction probe technology can detect double stranded DNA (double stranded DNA were T1 and its complementary DNA; although single stranded DNA are detected more sensitively for obvious reasons; see Figure 3.17). A segment of the gene encoding microtubule associated protein tau (MAPT) was chosen as the analyte for the SNP detection (Figure 3.18). Alleles of several SNPs of MAPT show association with increased cerebrospinal fluid levels of tau/ptau and might be linked to Alzheimer's disease.¹⁸⁸

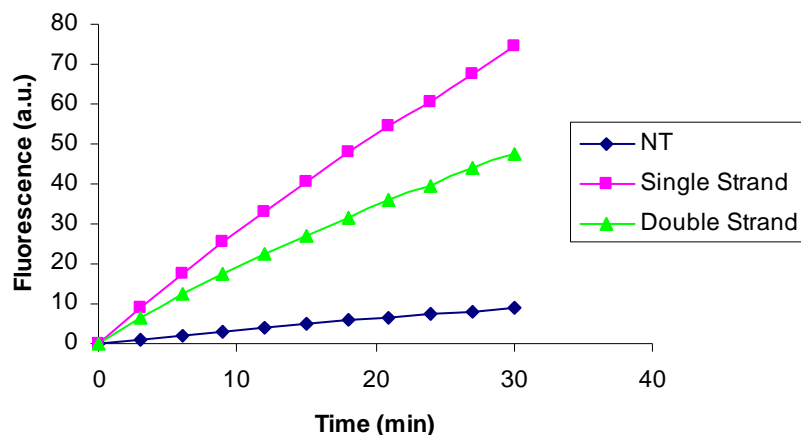


Figure 3.17 Detection of double strand DNA. Reaction conditions were the same as those for Figure 3.13.

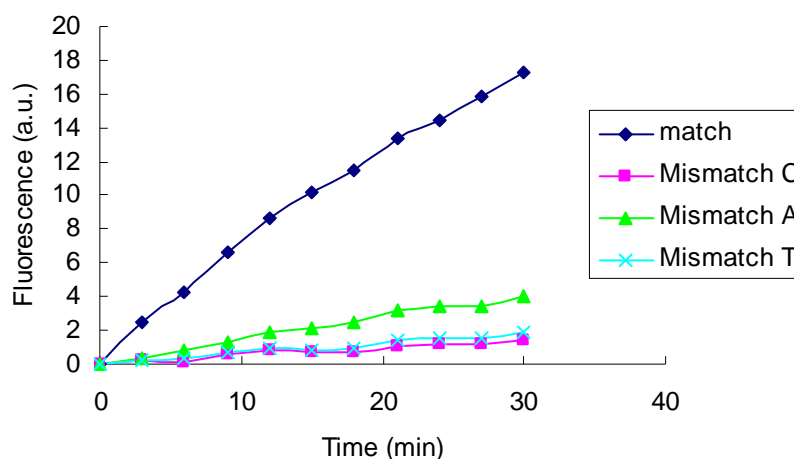


Figure 3.18 Detection of gene related to Alzheimer disease. Reaction condition: [probe A] = [probe B] = 50 nM, [template] = 10 nM, [BfuCI] = 0.1 U/ μ L, assay temperature = 30 °C. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 522$ nm, Ex slit = Em slit = 10 nM, PMT = 600 V).

3.10.2 RNA detection

The motivation of our program is to develop a non-PCR-based and an efficient platform that can detect RNA at isothermal conditions. So the detection of RNA was also pursued. Recently the ability of 223 type II restriction endonucleases to cleave RNA-DNA hybrid was examined.¹⁸⁹ Among them six restriction enzymes (AvaII, AvrII, BanI, HaeIII, HinfI and TaqI) are capable of catalyzing the robust and specific cleavage of both RNA and DNA hybrid strands.¹⁸⁹ This information will be useful when we expand the JP enzyme scope to detect RNA analytes in the future.

The levels of some microRNAs have been implicated in the etiology of cancers. For example decreased levels of miR-16 in patients' samples lead to an undesirable scenario whereby cancer cells have lower propensity to undergo apoptosis.¹⁹⁰

Therefore, the detection of miR-16 and related microRNAs levels in patients' tissue samples have important diagnostic values. The detection of microRNAs is non-trivial because of their comparatively short length and requires specially designed primers for effective RT-PCR detection assays of microRNA.¹⁹¹ Pleasingly, the junction probe technology can detect microRNAs such as miR-16 (see Figure 3.19).

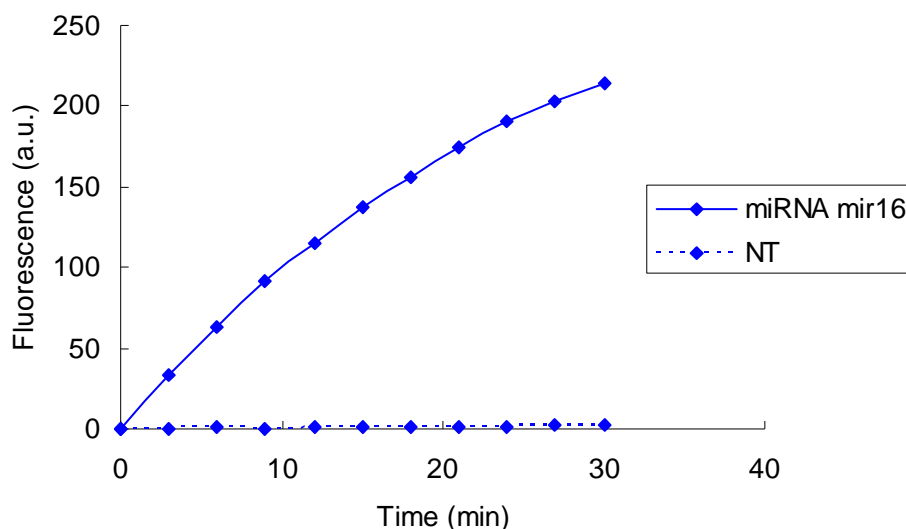


Figure 3.19 Detection of miRNA. NT = no template was added. [Probe A] = [probe B] = 1 μ M, [template] = 10 nM, [BfuCI] = 0.1 U/ μ L, assay temperature = 30 $^{\circ}$ C. Fluorescence data is normalized ($\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 522$ nm, Ex slit = Em slit = 10 nM, PMT = 600 V).

The JP technology can also detect ribosomal RNA in total RNA of *E. coli*; in this case, the intensity of the fluorescence signal corresponded with the quantity of total RNA (see Figure 3.20). The detection of *E. coli* with JP platform is specific because the probes that were specifically designed to detect *E. coli* failed to detect *V. harveyi* total RNA. Importantly, REase JP can detect total RNA in a bacterial cell lysate (see Figure 3.21) without the need for sophisticated sample clean-up.

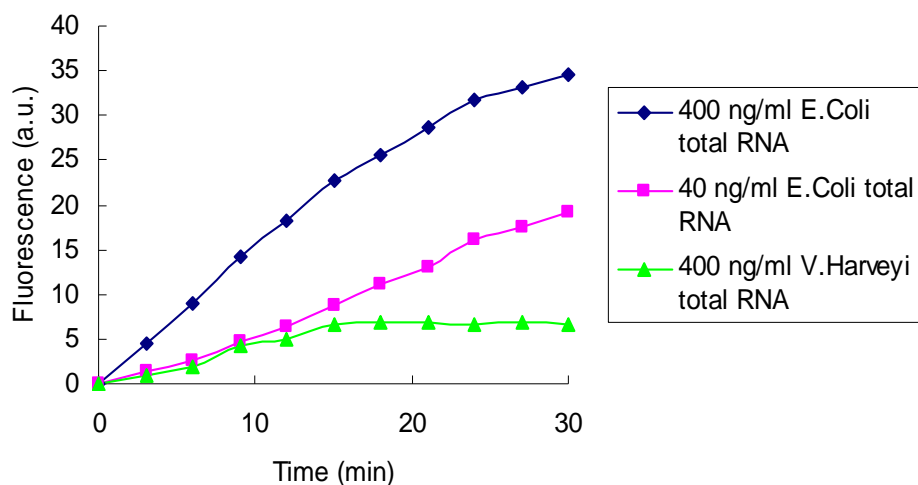


Figure 3.20 *E. Coli*-specific probes detect *E. coli* ribosomal RNA better than that of control RNA from *V. harveyi*. Reaction conditions were the same as those for Figure 3.13. Experiments were repeated 3 times and the error is less than 5%.

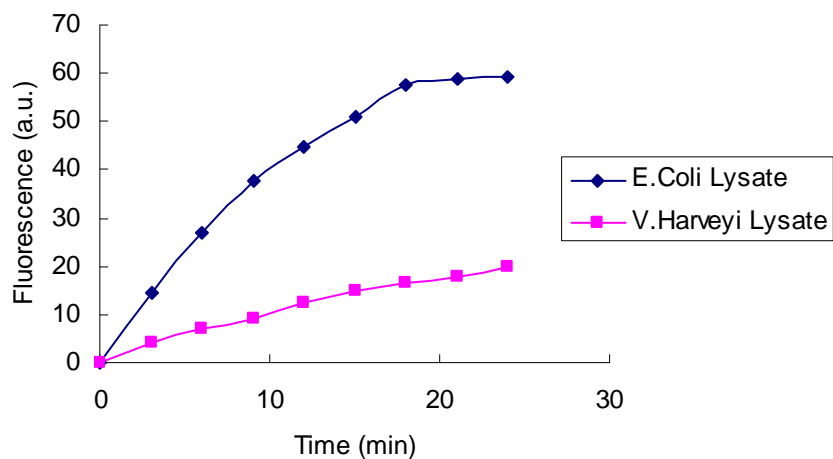


Figure 3.21 Ribosomal RNA in crude *E. coli* lysate was detected without separating out other macromolecules such the protein, RNA and carbohydrate debris. Of note, the crude lysate should not contain EDTA as Mg^{2+} is critical for the REase. Fluorescence data is normalized ($\lambda_{ex} = 494$ nm, $\lambda_{em} = 522$ nm). Reaction conditions were the same

as those for Figure 3.13. Experiments were repeated 3 times and the error is less than 5%.

Chapter 4: Sequences of nucleic acids and experimental methods

4.1 Sequences of nucleic acids

Table 4.1. Sequences of nucleic acids

<i>Template</i>	
T1	5'AGAGTCCACCAAGCTTCAGATTAG3'
T2	5'AGAGTCCACCAAGCTT <u>G</u> AGATTAG3'
T3	5'AGAGTCCACCAAGCTT <u>A</u> AGATTAG3'
T4	5'AGAGTCCACCAAGCTT <u>T</u> AGATTAG3'
T5	5'AGAGTCCACCAAGCTCTAGATTAG3'
T6	5'CAGGCAAGAGTCCACCAAGCTCTAGATTAG3'
	,
Complementary DNA to T1	5'CTAATCTGAAGCTTGGTGGACTCT3'
mir16	5'UAGCAGCACGUAAAUAUUGGCG3'
Targeted sequence on <i>E.coli</i>	5'...AGUCGACCGCCUGGGGAGUACGGCCGCAA
K12 MG1655 ribosome	GGUAAAAC...3' (16S RNA 873~910)
RNA	
Alzheimer's disease-related gene segment; perfect matching target	5'AGCAGTTGGCTTCGCCCAGGGTGCACCAGGACACGGTT3'
Alzheimer's disease-related gene segment; C mismatch	5'AGCAGTTGGCTTCGCCCAGGGT <u>C</u> CACCAGGACACGGTT 3'

SNP

Alzheimer's disease-related gene segment; A mismatch 5' AGCAGTTGGCTTCGCCCAGGGTACACCAGGA
CACGGTT 3'

SNP

Alzheimer's disease-related gene segment; T mismatch 5' AGCAGTTGGCTTCGCCCAGGGTTCACCAGGA
CACGGTT 3'

SNP

Probe A

A1 5'TCTGAAGCTGATCTTTT3'
A2 5'TCTGAAGCTTGATCTTTT3'
A3 5'TCTGAAGCTTTGATCTTTT3'
A4 5'TCTGAAGCT^{FAM[a]}TTGATC^{Dabsyl[b]}3',
A5 5'TCTGAAGCTT^{FAM}TGATC^{Dabsyl}3',
A6 5'TCTGAAGCTTT^{FAM}GATC^{Dabsyl}3',
A7 5'TAATCTGAAGCTTTGATC^{FAM}3',
A8 5'CTAGAGCT^{FAM}TTGATC^{Dabsyl}3',
A9 5'ATCTGAAGCT^{FAM}TTGATC^{Dabsyl}3',
A10 5'AATCTGAAGCT^{FAM}TTGATC^{Dabsyl}3',
A11 5' TAATCTGAAGCT^{FAM}TTGATC^{Dabsyl}3',
A12 5'CTAATCTGAAGCT^{FAM}TTGATC^{Dabsyl}3',
probe A for Alzheimer's disease related gene 5' GTGCACCCT^{FAM}TT GATC^{Dabsyl} 3',
probe A for mir16 5'CGCCAATATTT^{FAM}TTGATC^{Dabsyl}3',

probe A for *E. coli* 5'TGCGGCCGT^{FAM}TTGATC^{Dabsyl} 3'

ribosomal RNA

Probe B

B1 5'GATCATTGGTGGACTCTAAAA3'
B2 5'GATCAATTGGTGGACTCTAAAA3'
B3 5'GATCAAATTGGTGGACTCTAAAA3'
B4 5'AAAAGATCAAATTGGTGGACTCTAAAA3'
B5 5'AAGATCAATTTGGTGGACTCTAAAA3'
B6 5'AAGATCATATTGGTGGACTCTAAAA3'
B7 5'AAGATCTAATTGGTGGACTCTAAAA3'
B8 5'AAAGATCAAATTGGTGGACTCTAAAA3'
B9 5'AAGATCAAATTGGTGGACTCTAAAA3'
B10 5'AGATCAAATTGGTGGACTCTAAAA3'
B11 5'GATCAAATTGGTGGACTCTAAAA3'
B12 5'AAAAGATCAAATTGGTGGAC^{FAM}3'
B13 5'AAAA(S)^[c]GATCAAATTGGTGGACTCTAAAA3'
B14 5'AAAA(dS)^[d]GATCAAATTGGTGGACTCTAAAA3'
B15 5'AAAA(Ac)^[e]GATCAAATTGGTGGACTCTAAAA3'
B16 5'AAAA(OMe)^[f]GATCAAATTGGTGGACTCTAAAA3'
,
B17 5'GGGG(S)GATCAAATTGGTGGACTCTAAAA3'
B18 5'CCCC(S)GATCAAATTGGTGGACTCTAAAA3'
B19 5'TTTT(S)GATCAAATTGGTGGACTCTAAAA3'

B20 5'AGTAACGGAAAACCGTTACTAAAA(S)GATCAAA
TTGGTGGACTCT3'

B21 5'AGTAACGGAAAACCGTTACTAA(S)GATCAAATT
GGTGGACTCT3'

B22 5'TGTAACGGAAAACCGTTACA(S)GATCAAATTGG
TGGACTCT3'

B23 5'AGTAACGGAAAACCGTTACTAAAA(S)GATCAAT
TTGGTGGACTCT3'

B24 5'AGTAACGGAAAACCGTTACTAAAA(S)GATCATA
TTGGTGGACTCT3'

B25 5'TGTAACGGAAAACCGTTACA(S)GATCAATTTGG
TGGACTCT3'

B26 5'TGTAACGGAAAACCGTTACA(S)GATCATATTGG
TGGACTCT3'

B27 5'TGTAACGGTACACCATAAAAATGGTGTACCGTT
ACA(S)GATCATATTGGTGGACTCT3'

B28 5'TGTAACGGTACACCATAGAAGTCCTGAATCCTA
AAAAGGATTCAGGACTTCTATGGTGTACCGTTAC
A(S)GATCATATTGGTGGACTCT

B29 5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA
TTGGTGGACTCT3'

B30 5'TG(S)GATCGGCA(S)GATCGCAAAGC(S)TGCC(S)
GATCCA(S)GATCATATTGGTGGACTCT3'

B31	5'CA(S)GATCGCAAAGC(S)GATCTGTG(S)GATCG GAAAACC(S)GATCCA(S)GATCATATTGGTGGACTC T3'
B32	5'CT(S)GATCCGAAAACG(S)GATCAGCA(S)GATCG CAAAGC(S)GATCTGTG(S)GATCGGAAAACC(S)G ATCCA(S)GATCATATTGGTGGACTCT3'
B33	5'TG(S)GATCGGCT(S)GATCCGAAAACG(S)GATCA GCA(S)GATCGCAAAGC(S)GATCTGGA(S)GATCG GAAAACC(S)GATCTCCC(S)GATCCA(S)GATCATAT TGGTGGACTCT3'
B34	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TTGGTGGACTCTTGCCTG3'
B35	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TTGGTGGACTCTTGC3'
B36	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TTGGTGG3'
probe B for Alzheimer's disease related gene	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TGGGCGAAGCCA3'
probe B for mir16	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TACGTGCTGCTA3'
probe B for <i>E. Coli</i> ribosomal RNA	5'TG(S)GATCGGAAAACC(S)GATCCA(S)GATCATA TACTCCCCAGGCGGTCGACT3'

[a]. T^{FAM} = Fluorescein-conjugated dT. [b]. ^{Dabsyl} = Dabsyl group conjugated at 3' end. [c]. (S) = phosphorothioate linkage. [d]. (dS) = phosphorodithioate linkage. [e] (Ac) = phosphoroacetate linkage. [f]. (OMe) = methyl phosphate linkage.

4.2 Components of junction probes

Table 4. 2. Components of junction probes

	Template	Probe A	Probe B
JP1	T1	A2	B4
JP2	T1	A4	B8
JP3	T1	A4	B9
JP4	T1	A4	B10
JP5	T1	A4	B11
JP6	T1	A7	B12
JP7	T1	A4	B13
JP8	T1	A4	B14
JP9	T1	A4	B15
JP10	T1	A4	B16
JP11	T1	A4	B17
JP12	T1	A4	B18
JP13	T1	A4	B19
JP14	T1	A4	B20
JP15	T1	A4	B21
JP16	T1	A4	B22
JP17	T1	A4	B23
JP18	T1	A4	B24
JP19	T1	A4	B25
JP20	T1	A4	B26

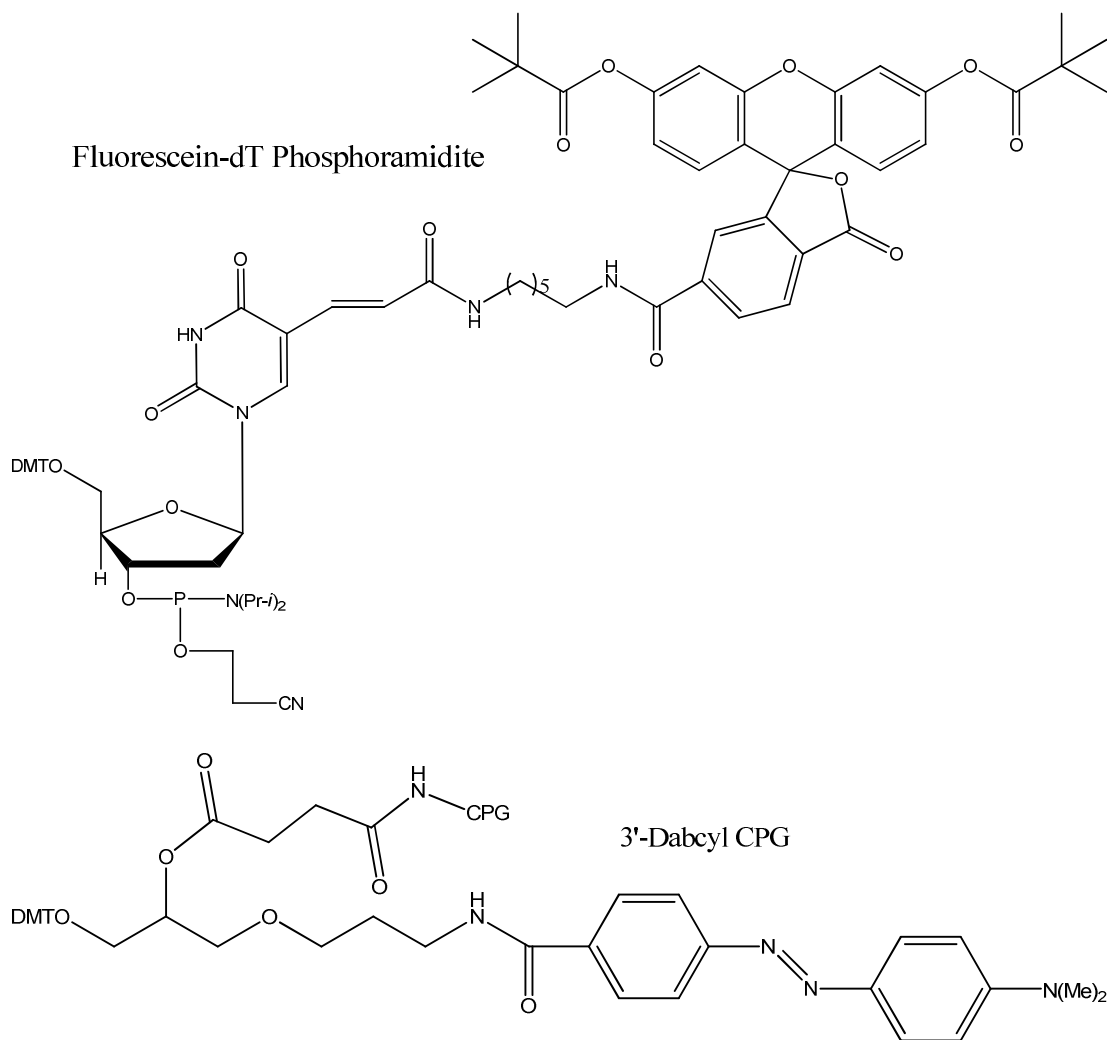
JP21	T1	A4	B27
JP22	T1	A4	B28
JP23	T1	A4	B29
JP24	T1	A4	B30
JP25	T1	A4	B31
JP26	T1	A4	B32
JP27	T1	A4	B33
JP28	T5	A8	B29
JP29	T1	A9	B29
JP30	T1	A10	B29
JP31	T1	A11	B29
JP32	T1	A12	B29
JP33	T6	A4	B34
JP34	T6	A4	B35
JP35	T6	A4	B29
JP36	T6	A4	B36

4.3 Materials and methods

4.3.1 Materials

Normal CPGs (dA, dT, dG and dC) and normal phosphoramidite (dA, dT, dG and dC) were purchased from Azco Biotech. Inc. Fluorescein-dT, 3'-dabsyl CPG, Pac-dA-Me phosphoramidite, dA-thiophosphoramidite, dA-PACE phosphoramidite, ultramild CE phosphoramidites (Pac-dA, Ac-dC and iPr-Pac-dG), sulfurizing reagent (Beaucage reagent), sulfurizing reagent II (DDTT), ultramild cap reagent mix A and Glen-PakTM cartridges were purchased from Glen Research. Restriction

endonuclease, BfuCI, its buffer (NE buffer 4 10X, 500 mM potassium acetate, 200 mM Tris-acetate, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9) and bovine serum albumin (BSA, 100X, 100 $\mu\text{g}/\text{mL}$) were purchased from New England Biolab[®], Inc. Staining reagent SYBR Gold was purchased from Invitrogen Corp.



4.3.2 Synthesis of oligonucleotides

All natural oligonucleotides were synthesized on 1 μmol scale on an Applied Biosystems (Model 392) synthesizer using standard β -cyanoethylphosphoramidite coupling protocol with DMT-on mode according to the manufacturer's manual.

Deprotection and cleavage of the oligonucleotides from the CPG support were carried out by incubating the CPG powders in ammonium hydroxide for 8 h at 55 °C. Synthesis, deprotection and cleavage of fluorophore labeled and unnatural oligonucleotides (modified with phosphoromonothioate, phosphorodithioate, phosphonoacetate, or methyl phosphate linkages) were synthesized on 0.2 μ mol scale on the same synthesizer using β -cyanoethylphosphoramidite coupling protocol with appropriate modifications according to manufacturer's protocols.

4.3.3 Purification of oligonucleotides

Oligonucleotides that are shorter than 40mer were purified by Glen-PakTM cartridge according to the manufacturer's manual. The others (fluorophore labeled or longer than 40mer) were first synthesized as "DMT-on" and purified by reverse-phase semi-preparative HPLC (Varian model 210 proster, Microsorb-MW 100-5C₁₈ column 250 x 10 mm) using solvent A (0.1 M triethylammonium acetate, pH 7.0) and solvent B (acetonitrile) (10% A to 30% A in 20 min, then 30% A to 100% A in 30 min) with a flow rate of 3 mL/min. The retention times of the labeled oligonucleotides were between 19-21 min. The HPLC-purified "DMT-on" oligonucleotides were then de-tritylated by Glen-PakTM cartridge according to the manufacturer's manual. All the purified oligonucleotides were dried in *vacuo* by Speedvac (Thermal Scientific, Model SAVANT DNA 120), and then dissolved in sterile H₂O.

4.3.4 Determination of oligonucleotide concentrations

The oligonucleotides were quantified by UV absorbance. The optical densities were measured at 260 nm by using a quartz cuvette with a 1 cm path length on a Jasco V-630 UV-Vis spectrophotometer. The sample concentration was calculated by using the oligo calculation software at www.idtdna.com.

4.3.5 Fluorescence analysis of the cleavage reaction

DNA Template, probe A and probe B in 60 μ L of NE buffer 4 1X (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) with Bovine Serum Albumin (BSA, 100 μ g/ml) were mixed at 30 °C, and the fluorescence at time = 0 min (referred to as t_0) was recorded. BfuCI was then added to the solution and fluorescence change was monitored. Fluorescence studies were performed on a Cary Eclipse fluorescence spectrophotometer. Unless specified, most reactions were monitored under these parameters: $\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 522$ nm, E_x slit = E_m slit = 5 nm, PMT = 600 V.

4.3.6 Gel-based analysis of the cleavage reaction

The reaction conditions were the same as described in general procedures. The 25 % denaturing polyacrylamide gel (8 cm x 10 cm x 1 cm) containing urea (7 M) was run under 250 V for 55 min in Tris borate-ethyleniamine tetraacetic acid buffer (pH 8.0). After electrophoresis the gel were stained with SYBR Gold stain. Images were obtained with STORM scanner (Molecular Dynamics).

4.3.7 Bacteria strains and RNA

Materials and reagents were sterilized by autoclaving at 120°C for 20 min. MicroRNA miR-16 was provided by Azco Biotech (San Diego, CA).

E. coli K12 MG1655 strain bacteria were incubated in Luria–Bertani (LB) medium on a shaker at 37 °C until OD₆₀₀ reached 0.77. 30 mL of the mixture was centrifuged at 5,500 g for 15 min at 4 °C. The supernatant was discarded. And the pellet was lysed in TE buffer containing lysozyme (10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.4 mg/mL lysozyme). Total RNA was extracted from the suspension by using a total RNA extraction kit according to the manufacture's manual. (<http://www.promega.com/tbs/tm279/tm279.pdf>)

V. harveyi MM32 strain bacteria were cultured the same way as *E. Coli* K12 MG1655 except in an autoinducer bioassay (AB) medium. AB medium preparation: The basal medium contained 0.3 M NaCl and 0.005 M MgSO₄. After adjusting the pH to pH 7.5 with KOH, it was sterilized by autoclaving (121 °C, 20 min). After cooling, the following sterile compounds were added: 1% (v/v) of 1 M potassium phosphate buffer pH 7.0 (K₂HPO₄/KH₂PO₄), 1% (v/v) of a 0.1 M-arginine solution, 2% (v/v) of a 50% (v/v) glycerol solution, and 2% (v/v) of a 10% (w/v) vitamin-free solution of casamino acids (Difco, Becton Dickinson & Co., Sparks, MD, USA). For solid media, 2% (w/v) agar (Difco) was added prior to autoclaving the basal medium.

Crude lysate sample for detection was obtained by first filtering 1mL of the supernatant of the TE lysate through a 3 kDa filter, and dissolving the biomolecules on the surface of the filter in 200 µL Dnase and Rnase free water.

Conclusion

REases are generally robust enzymes and have the potential to be used on crude samples without any extensive and costly sample preparation methods. Although the use of nucleases to detect nucleic acid has some precedent in the literature, detailed studies that have looked into how specific topologies affect the efficiency of nuclease-based detection methods are lacking. The development of isothermal amplification systems for the detection of bioanalytes will continue to attract interest. PCR has excellent sensitivity which is almost impossible to rival so these technologies are not meant to replace PCR-based methods but to complement PCR-strategies and to be used in scenarios where PCR is not appropriate due to either technical or financial reasons.

We have used the TeHyP concept to develop junction probes that can amplify DNA detection signals under isothermal conditions using REase. 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.

The detection of macromolecular analytes via TeHyP is emerging as a powerful and enabling technology. We have shown that small changes in the structure of nucleic acid sequences that lie next to restriction endonuclease recognition site can lead to several orders of magnitude change in the cleavage rate of the cognate recognition site. By gaining insights into the salient architectural features that affect the REase, BfuCI, cleavage rate, we have successfully engineered a new junction

probe technology that has a significantly shortened assay time (several orders of magnitude) and wider variety of analytes compared to the first generation junction probe technology. Importantly, the demonstration that RNAs and disease biomarkers in crude bacterial cell lysate can be detected without sophisticated sample preparation or purification by this new generation JP platform is significant and highlights the robustness of REases as well as justification for the development of detection technologies that utilize these enzymes. 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. This work should aid in future design of REase-based DNA/RNA diagnostic tools and takes us closer to the realization of a simple point-of-care detection platform.

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Curriculum Vitae

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