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REVIEW

DNA Strand Displacement Reaction: A Powerful Tool for Discriminating Single Nucleotide Variants

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Abstract

Single-nucleotide variants (SNVs) that are strongly associated with many genetic diseases and tumors are important both biologically and clinically. Detection of SNVs holds great potential for disease diagnosis and prognosis. Recent advances in DNA nanotechnology have offered numerous principles and strategies amenable to the detection and quantification of SNVs with high sensitivity, specificity, and programmability. In this review, we will focus our discussion on emerging techniques making use of DNA strand displacement, a basic building block in dynamic DNA nanotechnology. Based on their operation principles, we classify current SNV detection methods into three main categories, including strategies using toehold-mediated strand displacement reactions, toehold-exchange reactions, and enzyme-mediated strand displacement reactions. These detection methods discriminate SNVs from their wild-type counterparts through subtle differences in thermodynamics, kinetics, or response to enzymatic manipulation. The remarkable programmability of dynamic DNA nanotechnology also allows the predictable design and flexible operation of diverse strand displacement probes and/or primers. Here, we offer a systematic survey of current strategies, with an emphasis on the molecular mechanisms and their applicability to in vitro diagnostics.

Keywords DNA · Single nucleotide variants · Toehold-mediated strand displacement · Toehold-exchange reaction · DNA polymerases mediated primer extension reaction

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1 Introduction

A single nucleotide variant (SNV) is a genetic variation in a single nucleotide. SNVs occur at a frequency of 1 every 100–300 bases in the human genome, which may have important clinical consequences. Genetic SNV at coding regions are closely related to the causes of many somatic diseases such as cancer, Alzheimer's disease, and many others [1–4]. Therefore, disease-related SNVs found in the blood circulation, such as circulating tumor DNA (ctDNA) and microRNAs, may serve as important disease biomarkers for in vitro diagnosis, and can be used to monitor the development of the disease [5–9]. However, many important disease-related nucleic acids only present in trace levels. For example, the abundance of ctDNA was estimated to be only 1% or even 0.01% of the entire circulating DNA. Discrimination of SNVs in such low abundant nucleic acid markers is often challenged by interference from the large excess of wild-type counterparts. Therefore, techniques capable of discriminating rare SNVs with high sensitivity and specificity in liquid biopsies are highly desired and hold great promise for disease diagnosis and prognosis.

Current techniques for analyzing SNVs rely mainly on polymerase chain reaction (PCR) and nucleic acid sequencing [10–13]. However, both techniques are challenged by tedious and lengthy operation procedures, the need for expensive infrastructure and special expertise, and high error-rate for discriminating SNVs. The use of nucleic acid hybridization probes, such as molecular beacons [14, 15] and Taqman probes [16, 17], have helped improve assay speed and accuracy through exquisite Watson–Crick base pairing rules, but are generally difficult to design and operate, and extensive experimental validation and optimization are required. This is because the thermodynamic difference between a SNV and its wild-type counterpart is only a few kcal mol^{-1}, the discrimination of which requires a delicate balance between hybridization yield and sequence selectivity. Moreover, coexisting nucleic acids with high sequence similarity in liquid biopsies are often at concentrations 100–10,000 times higher than those of target SNVs. As high sequence interference may further comprise the analytical performance of hybridization probes, alternative strategies that are highly programmable and robust are thus urgently needed for the detection of SNVs over high abundance interfering sequences in real clinical samples.

Facing these challenges, DNA nanotechnology offers unique solutions for analyzing SNVs with high sensitivity, specificity, programmability and robustness. Specifically, DNA strand displacement reactions mediated by a short sticky end, known as the toehold, have shown remarkable tunability at both thermodynamic and kinetic levels [18]. As such, in silico sequence design becomes possible for generating strand displacement beacons or even more sophisticated strategies capable of isolating or enriching rare SNVs. DNA strand displacement can also be triggered or controlled using enzymes, which offer several unique mechanisms that facilitate signal amplification while maintaining high sequence specificity. In this review, we will summarize recent advances in SNV detection techniques that harness DNA strand displacement. We will first give a brief introduction of

the fundamental basis of varying strand displacement principles, and then discuss various SNV detection techniques based on three classes of strand displacement, including toehold-mediated DNA strand displacement, toehold-exchange, and DNA polymerase-mediated primer extension reaction.

2 Fundamental Basis of DNA Strand Displacement

Strand displacement reactions, such as toehold-mediated strand displacement and toehold-exchange, have been used widely as basic building blocks in DNA nanotechnology for the design of reconfigurable structures [19–21], logic gates, [22–26] DNA machines, [27–30] and biosensors [31–33] Compared with other nucleic acid hybridization probes, such as molecular beacons, strand displacement reactions offer much higher design-level and operation-level flexibility. Leveraging strand displacement reactions, diverse DNA nanostructures or devices can be tailored into powerful tools to amplify the sensitivity and specificity for the detection of SNVs. Here, we will first discuss the fundamental basis of various DNA strand displacement reactions with an emphasis on their potential for SNV analysis. Their applications in DNA nanotechnology have been reviewed elsewhere [34, 35].

2.1 Toehold-Mediated Strand Displacement

Toehold-mediated strand displacement was first introduced by Yurke et al. [36] in their seminal work in creating DNA tweezers. As shown in Fig. 1a, toehold-mediated strand displacement between a single-stranded input and a duplex substrate is initiated at a short complementary single-stranded domains on the substrate (referred to as toeholds), and progresses through a branch migration process that resembles a random walk. As more base pairs are formed, the reaction is thermodynamically favored. When the hybrid domain is short, the kinetics of strand displacement are determined mainly by the length and sequence of the toehold domain. Depending on the binding strength of the toehold (length and GC content), the rate constant may vary from 1 to 6×10^6 $M^{-1}s^{-1}$ [22]. When a point mutation occurs at or close to the toehold domain, it may significantly alter the kinetics of a strand displacement. Such a kinetic difference may be used to design assays that discriminate SNVs from wild-type counterparts. To guide the design of such assays, the rate of toehold-mediated strand displacement reactions in the presence of varying mutations can be predicted theoretically using an intuitive energy landscape model (IEL model) established by Srinivas and Winfree [37].

Toehold-mediated strand displacement also inspired the development of several other types of toehold designs capable of fine-tuning the thermodynamics or kinetics of strand displacement reactions. For example, the remote toehold, where a spacer is introduced between the toehold domain and branch migration domain, can be used to control the kinetics of strand displacement with a higher precision than the normal toehold (Fig. 1b) [38]. The allosteric toehold can increase the flexibility of regulating strand displacement reactions by splitting the toehold domain and the

Fig. 1 **a** Schematic of the toehold-mediated strand displacement reaction. Input *A* hybridizes with the complementary toehold of complex *X* to initiate branching migration, and then *A* displaces *B* to form a new complex *Y* [36]. **b** Schematic of remote toehold-mediated strand displacement. The toehold and displacement domains on both the duplex substrate and a single-stranded input strand are separated by spacer domains. Bounding of the duplex substrate and the input strand by hybridization of the toehold domains is followed by an internal diffusion step, initiating the branch migration reaction by which the short strand of the substrate is displaced [38]. **c** Schematic of allosteric toehold-mediated strand displacement. A short strand *R* first reacts with *CP* to form a reaction intermediate *CPR*, and the invading region of *R* exposes a short segment of *C* that serves as a secondary toehold to drive the strand-exchange between *X* and *CPR* to form *XC* and *RP* [39]. **d** Schematic of the toehold exchange reaction. Hybridization of the duplex substrate to the input *X* strand is initiated at the 5′ toehold of the substrate, proceeds through a branch migration process, and is completed via the spontaneous dissociation of the 3′ base pairs of the substrate to release single-stranded protector *Y*. The toeholds allow the forward and reverse reactions to proceed with fast kinetics [41]. **e** Schematic of the primer extension reaction mediated by DNA polymerases. Conformation of the probe is changed by hybridization of the probe with the target DNA, and then the target DNA is displaced in the process of the polymerization reaction and then hybridized to another probe [42]

branch migration domain (Fig. 1c) [39]. Such advanced toehold designs have also been adapted to create unique assays for discriminating SNVs [40].

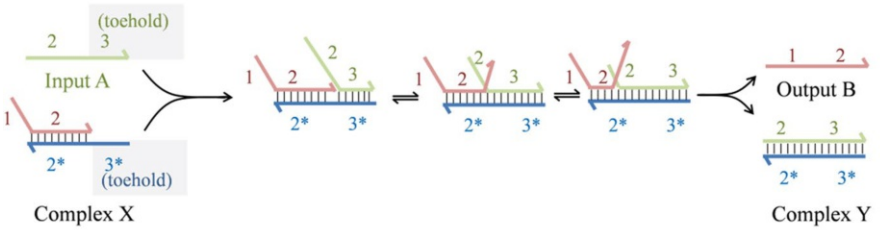
2.2 Toehold-Exchange

Of all toehold designs, toehold-exchange is one of the most powerful and widely used strategies for designing assays and probes that discriminate SNVs (Fig. 1d). This reaction, which was first introduced by Zhang et al. [41], involves a reverse toehold introduced into the duplex substrate. By simply tuning the length of the forward and reverse toehold, one can control the thermodynamics of the reaction with high precision. Moreover, the toehold-exchange principle also effectively decouples the thermodynamics and kinetics of the strand displacement, allowing the reaction to progress rapidly regardless of the thermodynamics. To better describe the kinetics of toehold-exchange, Zhang and Winfree [18] established a three-step reaction model, through which the kinetics can be predicted using a flow chart (Fig. 2). For a given toehold-exchange reaction, n and m represent the length of the forward and reverse toeholds, respectively. The reaction rate constant of this reaction can be determined roughly based on the numeric values of n and m. One of the most remarkable applications of toehold-exchange is to discriminate SNVs through fine-tuning the thermodynamics, i.e., the Gibbs free energy, of the reaction, which will be detailed in the section on SNV detection using toehold-exchange.

2.3 DNA Polymerase-Mediated Primer Extension Reaction

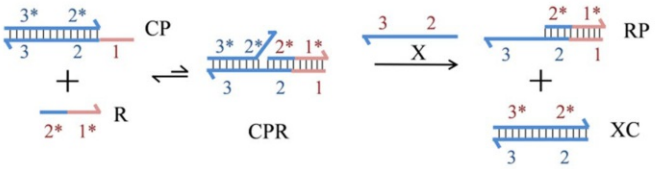
Stand displacement may also be triggered through a primer extension reaction mediated by DNA polymerases possessing high displacement activities (Fig. 1e) [42]. Enzyme-assisted DNA strand displacement has been used widely for isothermal nucleic acid amplification as well as for the development of enzyme-mediated DNA machinery. For example, strand displacement amplification that combines enzyme-assisted DNA strand displacement and a nicking endonuclease has been widely

A.Toehold mediate strand displacement

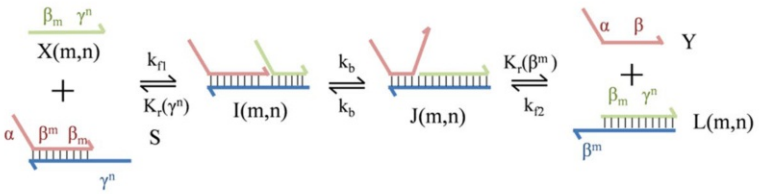


B. Remote toehold mediate strand displacement

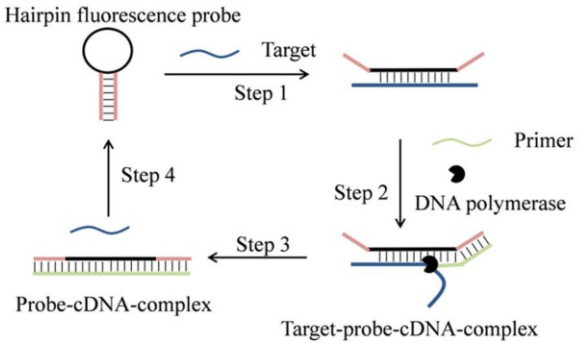
C. Allosteric toehold mediate strand displacement



D.Toehold Exchange mediate strand displacement



E. Primer extension reaction mediated by DNA polymerases



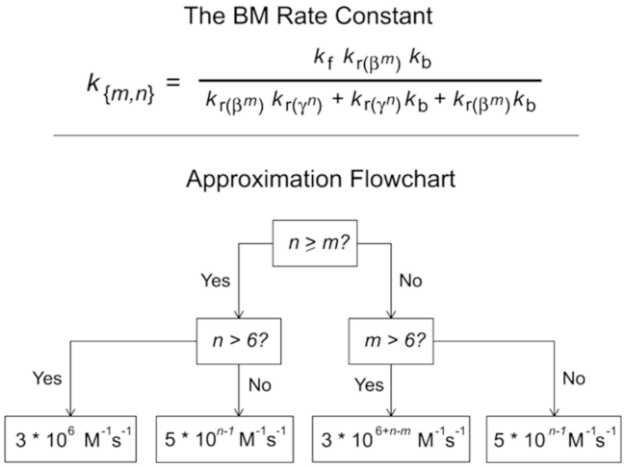


Fig. 2 Predicting the rate of toehold-mediated strand displacement reaction using a flow chart of three-step reaction model (reprinted with permission from [18])

adopted as an isothermal alternative to polymerase chain reactions [43, 44]. Further integration of strand displacement amplification with DNA hairpins also led to the development of beacon-assisted detection amplification [43]. Enzyme-assisted DNA strand displacement has also been incorporated as a signal amplification component into various assays for discriminating SNVs [45]. Sequence specificity can be achieved through the differential response to enzymes or through the integration of well-designed probes, primers, or other strand displacement techniques.

3 Discrimination of SNVs Using Strand Displacement Reactions

DNA strand displacement technology provides numerous tools for SNV detection, such as toehold-mediated strand displacement reactions, strand exchange reactions, and enzyme-assisted strand displacement reactions. These tools can be used to determine the presence or absence of SNV from a thermodynamic and kinetic perspective. Disease progression can be monitored by diagnosing the presence and concentration of SNV. The practical application of the above-mentioned related tools in SNV will be described in detail below.

3.1 SNV Detection Using Toehold-Mediated Strand Displacement Reaction

Current research on the detection of SNVs based on the strand displacement reaction relies mainly on reaction kinetics to achieve SNV detection [46–48]. When a point mutation occurs at, or near, the toehold region, the rate of strand displacement reaction is significantly reduced, thereby allowing the mutation to be distinguished from the wild type kinetically. Li [49] proposed a new SNV detection method using double-stranded probes to identify SNVs, improving the low specificity of direct

hybridization of single-stranded probes. The double-stranded probe overcomes the small difference in free energy between the single-stranded probe–wild type and the single-stranded probe–SNV direct binding, thereby enhancing the specificity of the recognition probe (see Fig. 3a, b). The length of the double-stranded probe toehold is also found to have a significant influence on the specificity of SNV recognition. When the toehold length is 6 or 7 nt, wild type and SNV can exhibit significant kinetic reaction rate differences, which is beneficial to the identification of SNV. For SNV detection in rare SNVs, Li introduced other double-stranded probes corresponding to the non-target SNV to shield from interference of the non-target SNV, thereby ensuring specific recognition of the target SNV (see Fig. 3c). Since then, SNV detection technology based on toehold-mediated strand displacement has mushroomed.

With the rapid development of the application of toehold-mediated strand displacement reaction in SNV detection, researchers were not satisfied with the SNV recognition specificity achieved by conventional linear probes. Therefore, how to improve the specificity of SNV detection method is now the focus of research. As shown in Fig. 4, most researchers studied probe structure with a view to increasing the difficulty of the strand displacement reaction between SNV sequences and probes by increasing the thermal stability of the probes, so as to enhance the difference in reaction kinetics between wild type and SNV and further improve specificity. Y-type [50] and X-type [51] probes are widely favored because their thermal stability is much higher than that of linear probes [52]. In addition to optimization of probe structure, locked-nucleic acid (LNA) can be used in strand displacement to improve the specificity of detection methods because

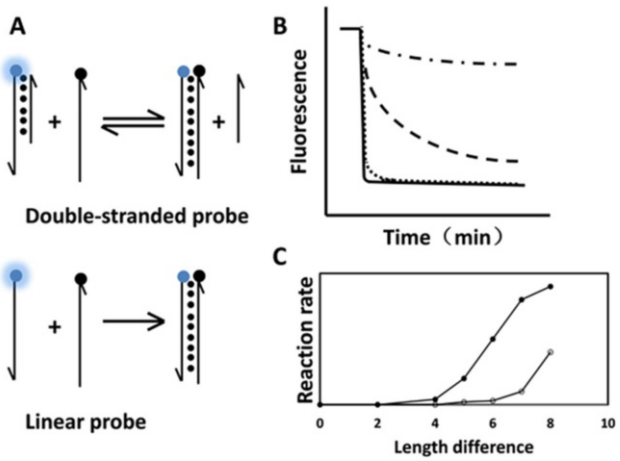


Fig. 3 **a** Labeling scheme for comparing double-stranded probe with linear probes. **b** Comparison of reaction kinetics of double-stranded probe with linear probes with matched and mismatched targets. While linear probes react equally with matched (*solid line*) and mismatched (*dotted line*) targets, double-stranded probes react well with matched (*dashed line*) but not with mismatched (*dot-dash line*) targets. **c** Dependence of the reaction rate on the difference in length between the long and short strands in the presence of the perfectly match (*solid circle*) and the single mismatch oligonucleotide (*open circle*) [49]

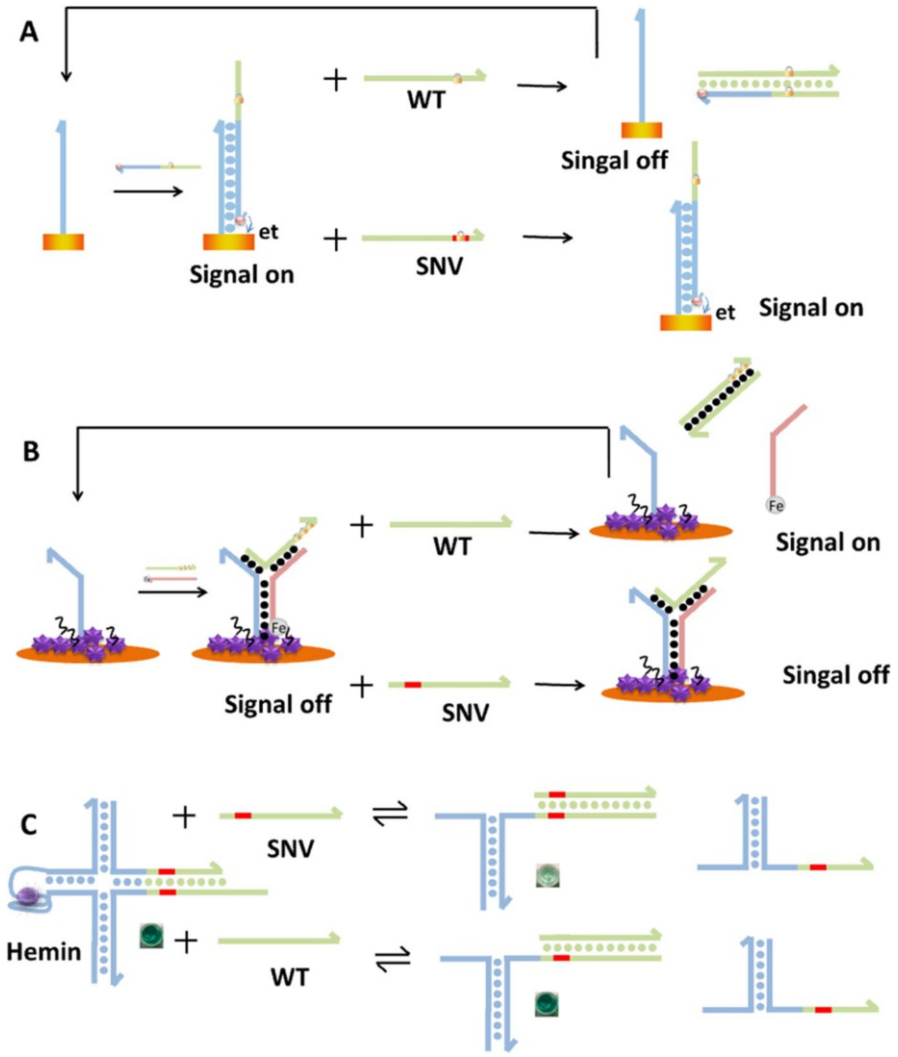


Fig. 4 Application of the double-stranded probe (**a**) [53], Y probe (**b**) [51], and (**c**) X-probe in single nucleotide variant (SNV) recognition [52]

of its function in amplifying the destabilization of mutations in double-stranded DNA. LNA contains a methylene linkage between the 2′-O atom and 4′-C atom of the ribose ring [53, 54], leading to restricted ribose conformations, resulting in a higher melting temperature of double-stranded with LNA [55, 56]. Therefore, the affinity between wild type and probe, and the repulsion between SNV and probe, can both be enhanced by replacing natural nucleotides with LNA at mutation sites. As a result, the reaction efficiency of the probe with the wild type was improved, while that with SNV was weakened, so as to expand the kinetic difference and enhance the specificity.

Typical toehold-mediated strand displacement reaction is limited by the toehold length of the hybridization probe. The toehold strand displacement rates of wild type and SNV are not much different when the toehold length is too long, resulting in poor recognition specificity. Therefore, Li et al. [57] proposed the one-sided remote toehold design based on a toehold-mediated strand displacement and internal diffusion driven branch migration mechanism [38] (Fig. 5a). In this design, a spacer region was added between the toehold and branch migration regions to introduce the internal diffusion step. In this way, one-sided remote toehold strategy increases the energy threshold of the strand displacement reaction to increase the difficulty of the reaction. By increasing the spacer length, the rate of the SNV reaction can be greatly reduced, while that of the complementary reaction changes little, thereby increasing the kinetic difference and the detection specificity.

A single binding event resulting from a single target binding domain of the probe may cause only small thermodynamic constraints for SNV. Compared with the overall reaction thermodynamics, this constraint has little effect on the reaction results of wild type and SNV, resulting in poor recognition specificity. Therefore, Liu et al. [58] increased thermodynamic limitations by designing probes with two

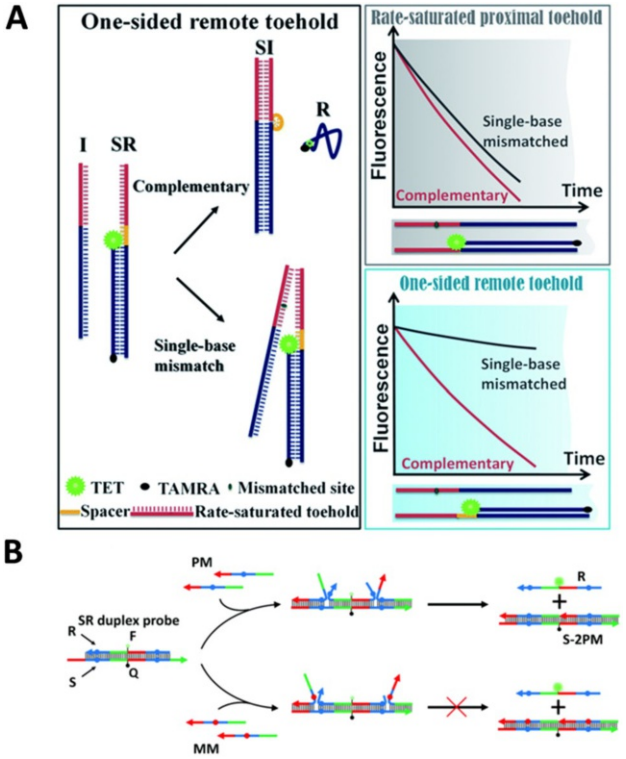


Fig. 5 **a** Schematic diagram of SNV discrimination based on the one-sided remote toehold-mediated strand displacement reaction response. Reprinted with permission from [57]. **b** Schematic diagram of SNV discrimination method based on cooperativity system (Reprinted with permission from [58])

target binding domains (as shown in Fig. 5b). Liu et al. [58] achieved the diagnosis of KRAS-G12D mutations by combining this design with PCR amplification. In this design, the SR double-stranded probe reacts with the target to achieve two binding events with synergistic effects [59, 60]. If the target contains SNV, two mismatched bubbles will be generated during the reaction, increasing the thermodynamic constraints. Thermodynamically, the probe with the double toehold has higher thermodynamic constraints, resulting in increased specificity. The system can also be combined with an isolation system to develop a highly specific dual discrimination strategy.

In addition to these tools, a framework nucleic acid (FNA) acting as a detection platform is also an important method to increase sensitivity and specificity. The probe-based FNA not only spatially segregates neighboring probes avoiding molecular entanglements but also molecular diffusion and convection is expected to be faster than at smooth macroscopic sites, which improves sensitivity of sensors greatly [61]. Zhang et al. [62] combined FNA with a DNA strand displacement reaction to develop a sensing platform for detecting SNPs. The probe marker with streptavidin captures the target nucleic acid by a strand displacement reaction, and, if a SNP is present, the process is aborted (Fig. 6a) [62]. Seeman et al. [63] also developed a DNA origami chip for SNP genotyping. This rectangular DNA tile harbors a pattern of alphabetic characters representing the four nucleotides (i.e., A, T, G, and C) that enable AFM imaging with a symbolic display. In the presence of complementary sequences, the corresponding probe can trigger DNA strand displacement and remove the symbolic character while other toehold-based migrations would be inhibited by a single-base mismatch (Fig. 6b).

Moreover, a defect of the toehold-mediated strand displacement reaction method is that it can judge only whether nucleic acids have a mutation, but not mutation location. To solve this problem, Liu et al. [39] proposed using the logic judgment function of DNA logic circuit to identify the location of SNV. As shown in Fig. 7a, The 4-to-2 encoder was constructed based on the allosteric toehold-mediated strand displacement reaction, which can be used to specifically determine SNVs and their positions [64]. There are two recognition elements in the encoder, B-C and G-L, which distinguish mutations of region 1* and 2* and region 2* and 3* of the sequence, respectively. After the SNV sequence is recognized by the circuit, the encoder will output different signal combinations according to the region where the SNV site is located. As Fig. 7b shows, when the first number is 1, the analyte has no SNV in domains 1* and 3*. When the second number is 1, the analyte has no SNV in domains 3* and 2*. Thus, SNV sites can be located precisely in an exact domain. Although this circuit is a useful SNV recognition circuit, it has some limitations in the recognition of rare SNVs. Therefore, the circuit is hopefully cascaded with the amplification circuit to achieve a lower detection limit.

3.2 SNV Detection Using Toehold-Exchange

Although the toehold-mediated displacement reaction can be used to differentiate SNVs, it suffers from the limitation that the SNV needs to be in the vicinity of the

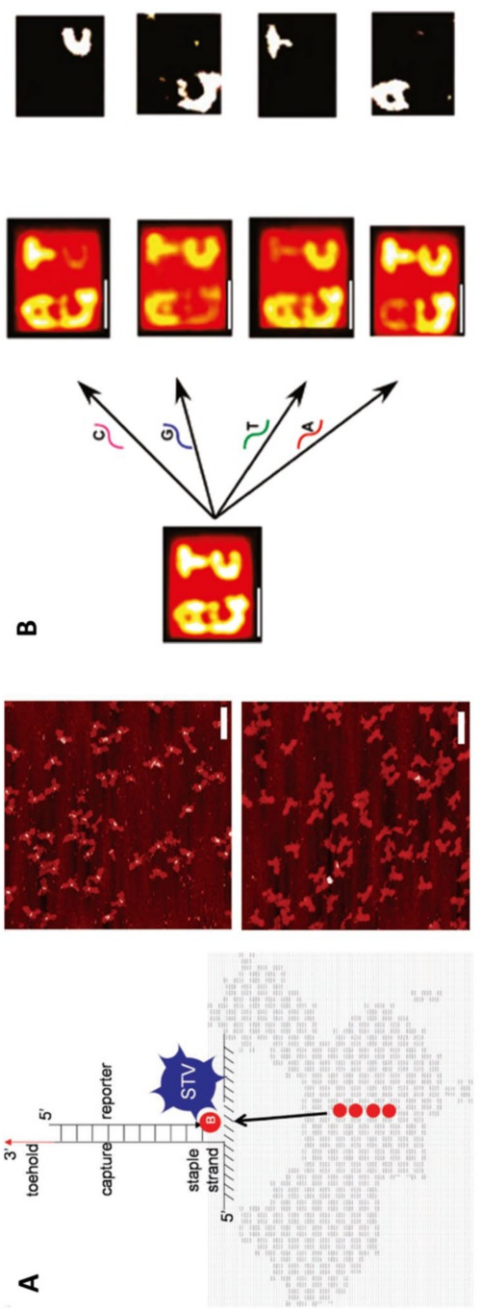
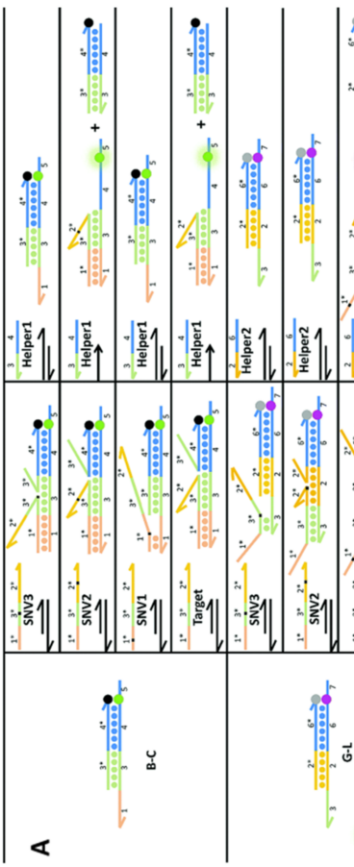


Fig. 6 **a** DNA framework nucleic acid (FNA) origami-based toehold-mediated SNP detection. Reprinted with permission from [62]. **b** Alphabetic patterned origami structure for SNP detection (reprinted with permission from [63])



	INPUTS				OUTPUTS	
	D0	D1	D2	D3	X	Y
SNV3	1	0	0	0	0	0
SNV2	0	1	0	0	1	0
SNV1	0	0	1	0	0	1
TARGET	0	0	0	1	1	1

Fig. 7 **a** SNV recognition method based on a 4-to-2 encoder. **b** Truth table of the 4-to-2 encoder (reprinted with permission from [64])

toehold area. This strand displacement reaction also shows poor performance on SNVs that are very close in thermodynamic energy to the wild type. These limitations can be overcome by probes operated using toehold-exchange principles [65]. The reversible nature of toehold-exchange makes it very sensitive to subtle changes in thermodynamic difference caused by SNVs. Reaction yield and discrimination factors (DFs) can be predicted accurately and tuned by altering the lengths of the forward and reverse toeholds. It is also possible to further improve the specificity for discriminating rare SNVs by introducing competitive "sink" probes [66]. This "sink" strategy takes advantage of both kinetics and thermodynamics to enhance hybridization specificity, making it possible to detect a minute amount of SNVs with a large excess of wild-type interference. Zhang et al. [65] achieved in vitro diagnosis of mutant nucleic acids such as EGFR-L858R by this strategy with a large excess of wild-type interference.

The fundamental basis of discriminating SNVs using toehold-exchange was first established by Zhang and colleagues in 2012 (Fig. 8) [65]. The standard Gibbs free energy (ΔG°) of a toehold-exchange reaction between an input X and duplex CP was found to be tunable by altering the length of the forward and reverse toehold. When ΔG° is highly negative, this reaction has a high reaction yield but low sequence specificity (DF is close to 1). However, when ΔG° is tuned to be near 0, the reaction yield approaches 50% and the DF increases dramatically. Hybridization probes making use of toehold exchange were also found to be highly robust, and resistant to changes in assays conditions such as buffer saline and temperature. In addition to toehold length, it is also possible to further fine-tune the toehold-exchange through auxiliary probes such as the protector probe (*P*) in Fig. 8. Because thermodynamic parameters of toehold-exchange probes are readily available, it is possible to achieve the in silico design of such probes without the need for experimental optimization. Such simulation-guided designs and operations of toehold-exchange probes make them highly attractive for practical application in SNV analyses. Zhang et al. [65] detected the let 7 g and other SNV nucleic acids through this design.

One inherent restriction of typical toehold exchange reaction is that the hybridization probes can only recognize single-stranded targets with mutations sites. Therefore, Zhang et al. further expanded the target to double-stranded nucleic acids through the development of an X-probe (Fig. 9) [67]. In this design, two different toeholds are created at the same end of each complementary strand in a duplex. Upon binding with the probe, a quadruplex DNA structure will be formed. If the target contained a base pair of SNVs, two mismatch bubbles will be generated in the produced duplexes. Thermodynamically, SNVs in the double-toehold exchange reaction are less favorable than that in a single-toehold counterpart, resulting in the enhancement in specificity.

Despite excellent performance for discriminating SNVs using toehold-exchange, it remains analytically challenging to detect rare SNVs in the presence of a large excess of somatic nucleic acids. To address the need for discriminating rare mutations, Zhang et al. [66] introduced a "sink" strategy, which dramatically improved the specificity of toehold-exchange probes. Because of the complexity in the reaction network, they built a model to simulate and predict the yield and DF distribution as a function of probe and sink reaction free energy. The basic principle is to

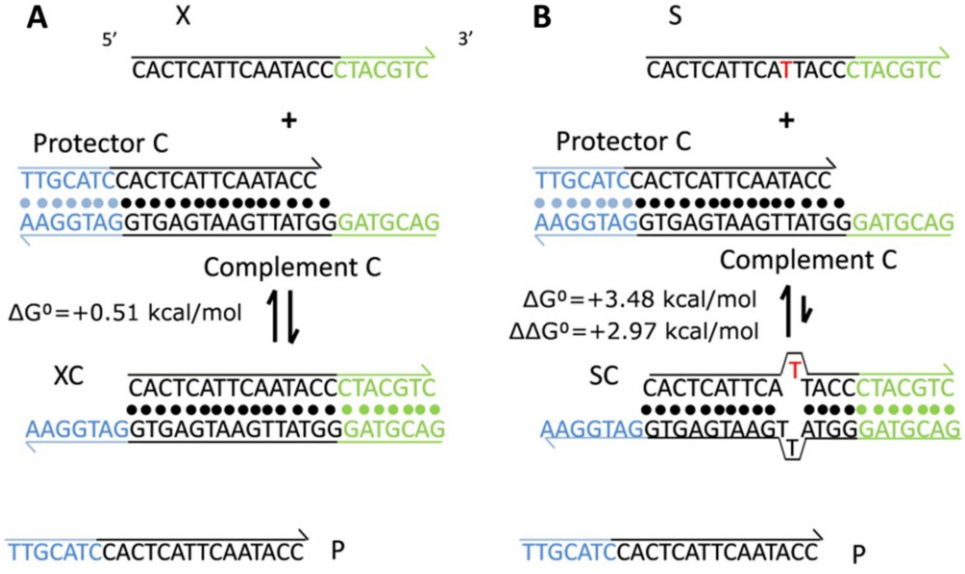


Fig. 8 Toehold exchange probes for SNV identification. **a** The probe reacts with an incorrect target *X* to release protector strand *P*. In this process, the standard free energy of the reaction is close to zero. **b** Hybridization of a spurious target *S* with one base change is thermodynamically less favorable by +2.97 kcal mol^{-1} [65]

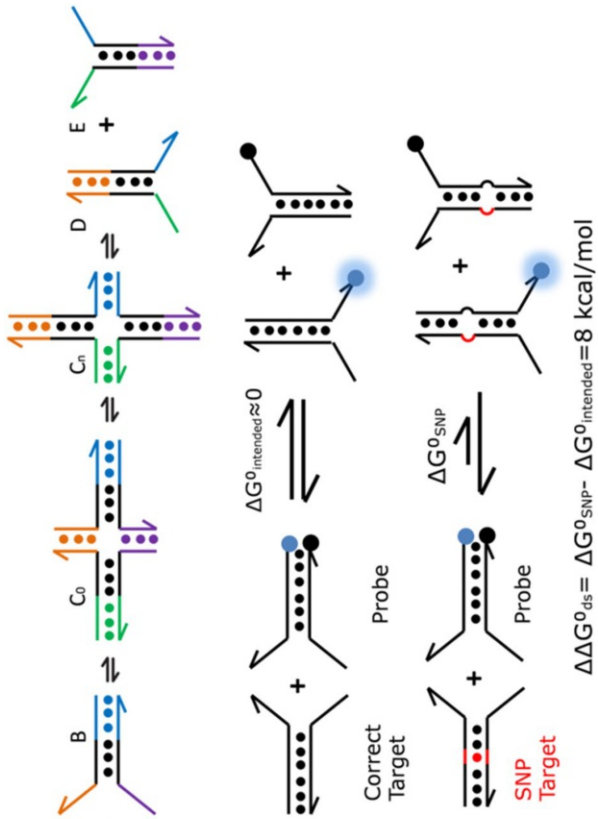


Fig. 9 Schematic representation of the double-stranded toehold exchange mechanism. **a** Reversible branch migration process of double-chain toehold exchange. **b** Highly specific variants identification based on double-stranded toehold exchange. The reaction with the intended target has $\Delta G^{\circ}_{\text{intended}}$ is close to zero. The reaction between the probe and the SNP target will result in two mismatch bubbles, and the reaction $\Delta G^{\circ}_{\text{SNP}}$ will be about 8 kcal mol^{-1} [67]

reduce the reaction potential between unexpected spurious targets and signal-generating probes by splitting the target-probe hybridization process. The perfect match target-probe pairs have faster kinetics than that with a SNV mismatch. Therefore, unwanted wild-type target will be annihilated by the sink instead of competing the SNV target to generate a signal. Seelig et al. [68] further improve the sensitivity of the Sink design by introducing two fuel strands (Fig. 10). Using this strategy, both the sensitivity and specificity can be enhanced. In addition to linear probes, DNA hairpins may also be used as competitive "sink" probes to enhance the sequence

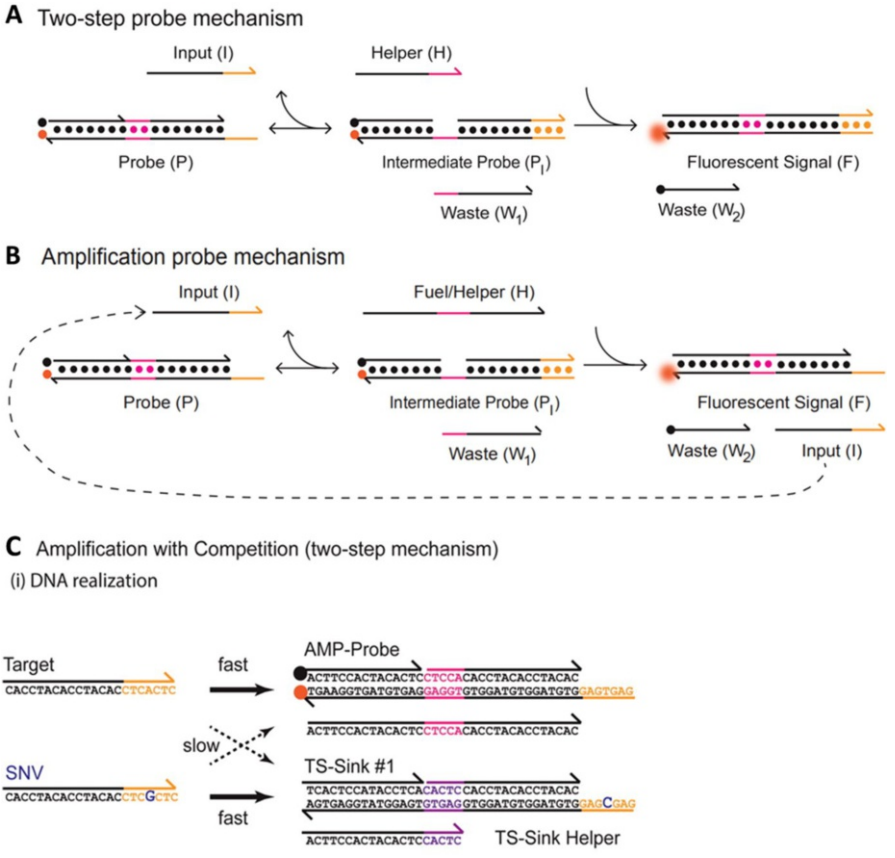


Fig. 10 Using an engineered two step sink and catalytic probe, SNVs in any position of the input can be reliably identified. **a** Two-step reaction mechanism. The first reaction step is a reversible toehold exchange reaction between the input strand (*I*) and the waste strand (*W1*). In the second step, an auxiliary helper molecule (*H*) competes with *W1* for binding to the magenta toeholds. Binding of *H* makes the overall reaction irreversible. **b** An amplification probe mechanism. Binding of the input strand is reversible, exactly as for the two step probe. In the second reaction step, the longer helper (or fuel) sequence hybridizes to the bottom strand using the pink toehold and displaces not only the waste strand *W2* but also the input irreversibly. After the input is released, it can react with another probe to initiate the next catalytic cycle. **c** An amplification circuit with a competitive two-step sink exhibits dramatically increased specificity and sensitivity for SNVs (reprinted with permission from [68])

specificity [69]. For example, Liu and coworkers combined hairpin competitor and asymmetric PCR amplification with in vitro diagnostics. Eventually, they successfully detected mutant nucleic acids (KRAS-G12D) with only 0.2% abundance [58] (Fig. 11).

When discriminating SNVs using toehold-exchange reaction, a tradeoff between assay sensitivity and specificity often occurs. Integration of toehold-exchange probes with nucleic acid amplification can help address this intrinsic trade-off. PCR and isothermal nucleic acid amplification was often used to pre-amplify the target sequence before the SNV analysis. It is also possible to amplify the detection signal during toehold-exchange process by introducing catalytic DNA circuits. Recently, Li and coworkers [70] explored the possibility of post-SNV-recognition amplification through the integration of toehold-exchange with a three-dimensional (3D) DNA nanomachine (Fig. 12). The 3D DNA nanomachine was designed by co-conjugating a toehold exchange probe with hundreds of signal reporters on a single gold nanoparticle. In the presence of the target, the nanomachine was activated through toehold-exchange and then cleaved signal reporters processive through nicking recognition and cleavage. As a result, each target could trigger the release of hundreds of fluorogenic signal reporters and the signal could thus be amplified by over 100 times. This strategy was shown to be fully compatible with toehold-exchange, allowing simulation-guided sequence designs and high sequence specificity. In addition to enzyme-powered DNA nanomachine, signal amplifications can also be achieved through enzyme-free strand displacement networks, which will be detailed in the next section.

3.3 Amplified SNV Detection Using Enzyme-Free Strand Displacement Networks

An enzyme-free strand displacement network [71] is often used in conjunction with strand displacement probes to amplify detection signals to address the trade-off in sensitivity and specificity [40, 72, 73]. The target can be used as a promoter for the amplification network or recognized by a recognition mechanism to induce an amplification mechanism. Both can amplify the target signal and distinguish it from SNV. For example, catalytic hairpin assembly (CHA), as an isothermal and enzyme-free signal amplification DNA network, plays an important role in recognition of SNV due to its remarkable inhibition of catalytic activity in the catalytic strand [74]. As shown in Fig. 13a, in the early stage of the reaction, the wild type as the catalytic strand undergo a CHA reaction, rapidly amplifying the signal in a short time, and expanding the signal difference with the SNV, thereby increasing DF [71]. Signal amplification can be achieved through target recycling. Similarly, Chen et al. [73] also achieved signal amplification by re-releasing the target into the DNA tetrahedron, which was based on FNA, thereby achieving an increase in specificity and sensitivity (as shown in Fig. 13b). The recognition probe modified on the tetrahedron limits the freedom of single-stranded DNA, providing a rigid platform to facilitate the strand displacement amplification process. In addition to the reuse of targets on an amplification network, mass amplification has also been used as a novel amplification method (Fig. 13c). In this design, only wild type can be captured

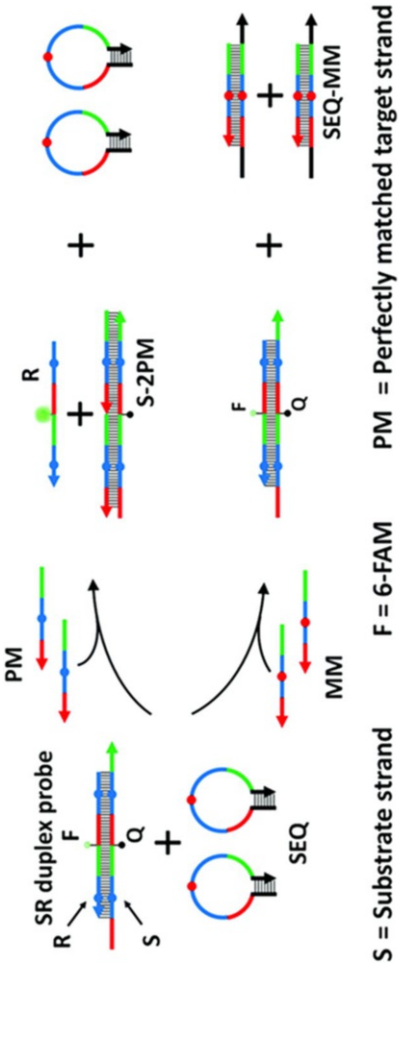


Fig. 11 Schematic representation of the proposed SNV discrimination methods based on cooperativity and combining cooperativity with sequestration (reprinted with permission from [58])

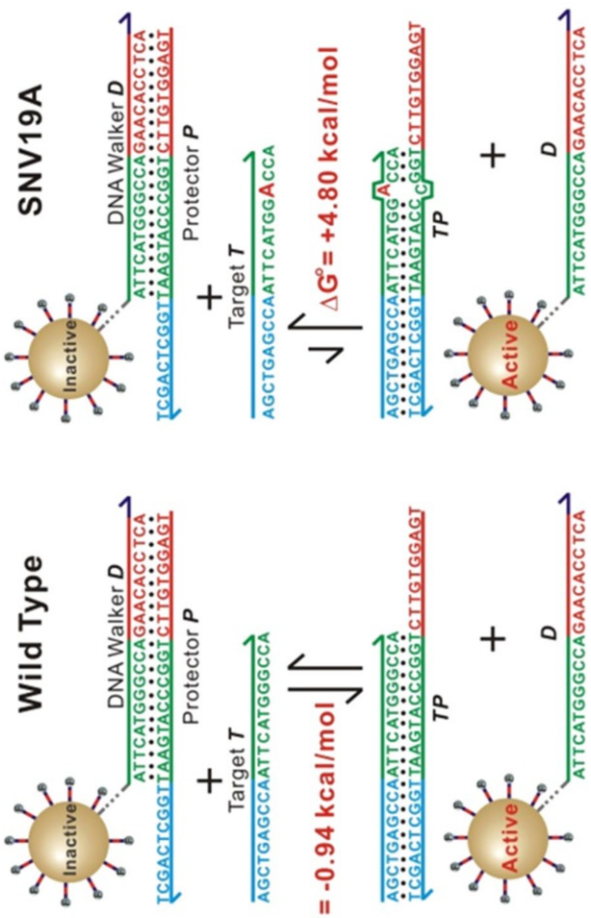


Fig. 12 Enhancing both sensitivity and specificity for discriminating SNVs by integrating toehold-exchange with a three-dimensional (3D) DNA nanomachine [70]

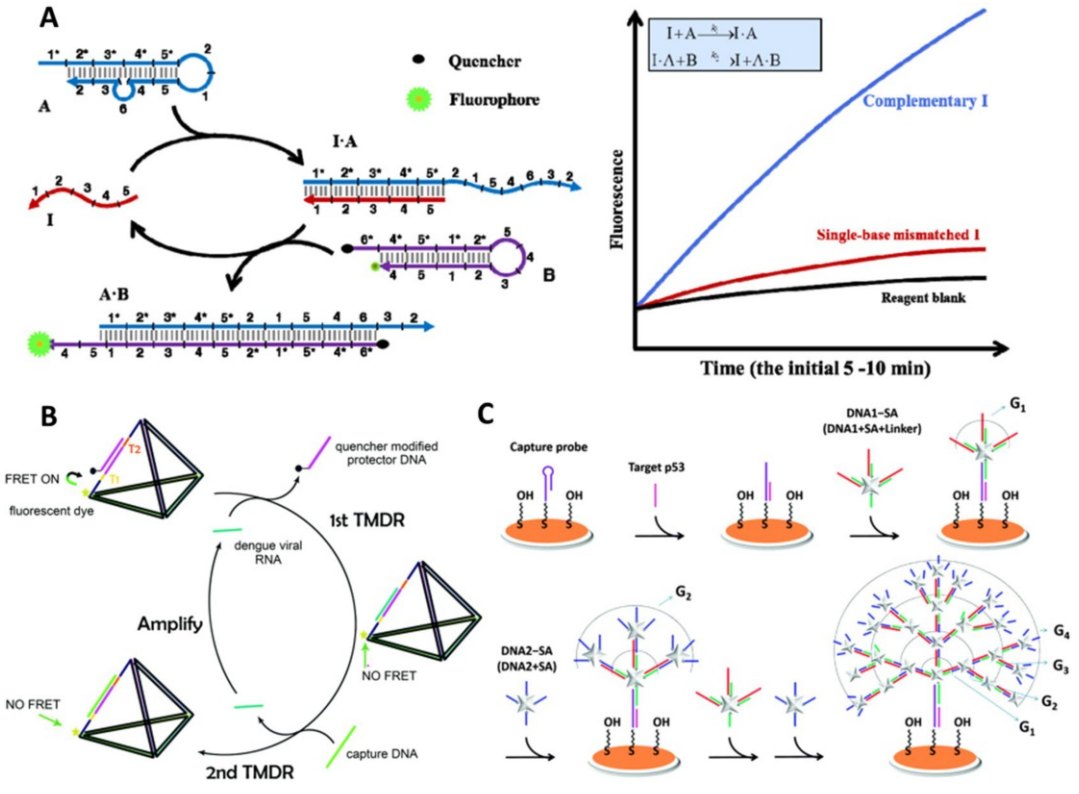


Fig. 13 **a** Schematic diagram of SNV recognition based on the initial reaction rate of the CHA circuit. Reprinted with permission from [74]. **b** Schematic diagram of SNV detection method based on the cascade of strand displacement and strand displacement amplification technology. Reprinted with permission from [73]. **c** Schematic diagram of SNV detection principle based on DNA-SA dendrimer nanostructure-amplified quartz microbalance sensing platform. Reprinted with permission from [75]

by probes modified on the quartz crystal microbalance to assemble dendrimer nanostructures, increasing mass and changing the signal frequency shift response [75]. Among them, Streptavidin's special four binding sites allow one DNA1-SA to bind to three DNA2-SAs, and one DNA2-SA can be binded to three DNA1-SAs, so the probe mass is increasing violently, achieving amplification. Furthermore, the complementary target signal is significantly amplified and the SNV signal does not, which result in significant kinetic signal differences and increases DF. The use of this enzyme-free strand displacement amplification network can improve the sensitivity and specificity of the recognition technology, which has broad application prospects in SNV applications.

3.4 SNV Detection Using DNA Polymerase-Mediated Primer Extension Reaction

DNA polymerase-mediated primer extension reaction can be used as a signal amplification component or as a terminal mutation recognition component, which plays an important role in SNV detection [45, 76–78]. The reaction was originally proposed by Guo et al. [42] and the relevant principles were clarified in 2009. DNA polymerase-mediated primer extension reaction is divided into four steps. Steps 1 and 2 are commonly used for SNV detection. As shown in Fig. 14a, when the target is identified based on step 1, the wild-type DNA can fold the hairpin and the signal cannot be recovered [44]. The mutant DNA can unfold the hairpin to initiate an enzyme-assisted strand-displacement reaction, which in turn releases the target and reuses it for signal amplification. Based on isothermal strand-displacement polymerase reactions, Liu et al. [45] realized the detection of mutations R156H and R156C which will be causing epidermolytic hyperkeratosis (EHK). This further extends the signal difference between the SNV and the target. When the SNV is identified based on the step 2, the target to be tested is usually set as a primer (Fig. 14b). At this time, a polymerase such as Klenow enzyme functions. Such enzymes have the $5' \rightarrow 3'$ polymerase activity and $3' \rightarrow 5'$ exonuclease activity of normal polymerases, but lack the $5' \rightarrow 3'$ exonuclease activity of intact polymerase. This means that such enzymes cannot cleave mutant bases when they encounter an SNV site during polymerization, so that polymerization still occurs but the reaction rate becomes extremely slow, forming a significant kinetic difference with the complementary target [79]. This special property makes the detection of terminal mutations feasible. In addition, the polymerase-assisted strand displacement reaction can also form a two-step recognition mechanism with the toehold exchange reaction, which has high specificity recognition and can achieve low abundance detection as low as 0.3% within 5 min (Fig. 14c). In this system, DNA polymerase-mediated primer extension reaction calibrates the products of the first-order discrimination reaction and determines whether to continue the reaction [80]. Since the products of the first-order discrimination reaction of the non-target nucleic acid are not completely complementary, when the reaction proceeds, it is terminated by incomplete matching. This mechanism allows SNV to cause significant deviations in conversion rates and achieve high specificity for low abundance detection. Zhao et al. [77] detected mutant nucleic acids (BRAF V600E) with only 1% abundance using this mechanism.

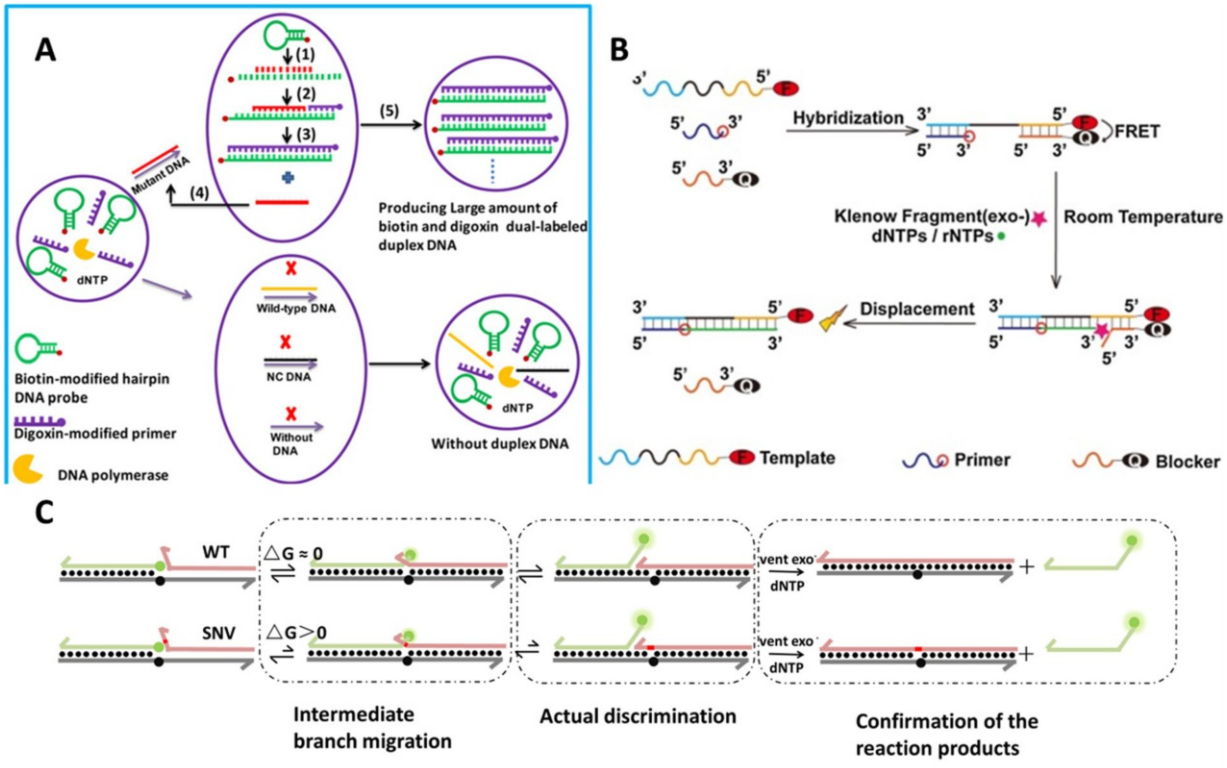


Fig. 14 **a** The schematic diagram of target DNA signal amplification based on the DNA polymerases mediated primer extension reaction. Reprinted with permission from [44]. **b** The scheme of using fluorescence resonance energy transfer method to measure primer extension kinetics catalyzed by Klenow fragments (exo−). The *red circle* highlights the single nucleotide substitution in the primer sequence. Reprinted with permission from [79]. **c** The schematic diagram of a two-step recognition mechanism combines toehold exchange reaction and the DNA polymerases mediated primer extension reaction [80]

Table 1 Discrimination factor (DF) values of different mutation identification methods

Discrimination format	Discrimination factor (DF)	Reference
Toehold-mediated strand displacement		
Quartz crystal microbalance biosensor based on toehold mediated strand displacement reaction	63 (G > C substitution)	[46]
Amplified quartz crystal microbalance platform	17–72 (3 SNVs)	[75]
Competition and catalytic amplification	23 (A > G substitution)	[68]
Combining cooperativity with sequestration	67–618, median = 194 (12 SNVs)	[58]
DNA polymerases mediated primer extension reaction		
The droplet-based microfluidic platform	100–550	[79]
Toehold exchange mediated strand displacement		
Competitive and amplification assisted by exonuclease III	33–44	[81]
Energy driven cascade recognition	45–109, median = 70 (6 SNVs)	[82]
A branch-migration based fluorescent probe	89–311	[80]
Enzyme-powered 3D DNA nanomachine	7–26, median = 12 (9 SNVs)	[70]
Conditionally fluorescent molecular probes	Median = 43	[67]
Simulation-guided DNA probe and sink design	Median β = 890c (44 SNVs)	[66]
Optimizing the specificity of nucleic acid hybridization	3–100, median = 26	[65]

3.5 The performance evaluation index of SNV detection method

Specificity and sensitivity are commonly used as indicators of detection in SNV detection. Sensitivity, or detection limit, refers to the lowest analytical concentration detectable by the detection system. Specificity is usually assessed using the DF, indicating the discrimination degree of the method against false targets. The calculation formula of specificity and sensitivity is as follows: [66]

$$\text{Specificity (DF)} = \frac{\chi_{\text{T}}}{\chi_{\text{N}}} \cong \frac{\frac{[T\cdot\text{Probe}]}{[T]_0}}{\frac{[N\cdot\text{Probe}]}{[N]_0}} \text{or DF} = \frac{\chi_{\text{T}}}{\chi_{\text{N}}} \cong \frac{\Delta \text{F}_T}{\Delta \text{F}_N},$$

$$\text{Sensitivity(yield)} = \frac{[T\cdot\text{Probe}]}{[T]_0}$$

where T and N represent the target and non-target molecules, respectively. χ is the hybridization yield. $[T]_0$ and $[N]_0$ represent the initial concentration of T and N molecules, respectively. $[T\cdot\text{Probe}]$ and $[N\cdot\text{Probe}]$ represent the product concentration of T and N molecules reaction, respectively. ΔF is the net increment of the signal.

The literature recognizing mutation based on strand displacement reactions and explicitly calculated DF values is listed in Table 1.

Although the DF index can be applied to most scenarios, there is an error in the evaluation of DF in actual sample detection. Because the SNV target usually has a low abundance, the tradeoff between sensitivity and specificity becomes extremely

significant. In order to solve this problem, Zhang et al. [66] introduced a new metric, the normalized multiple change β, mathematically expressed as:

$$\beta \cong \frac{(T \cdot \text{Probe})/\text{VAF}}{\text{background} + (N \cdot \text{Probe})}$$

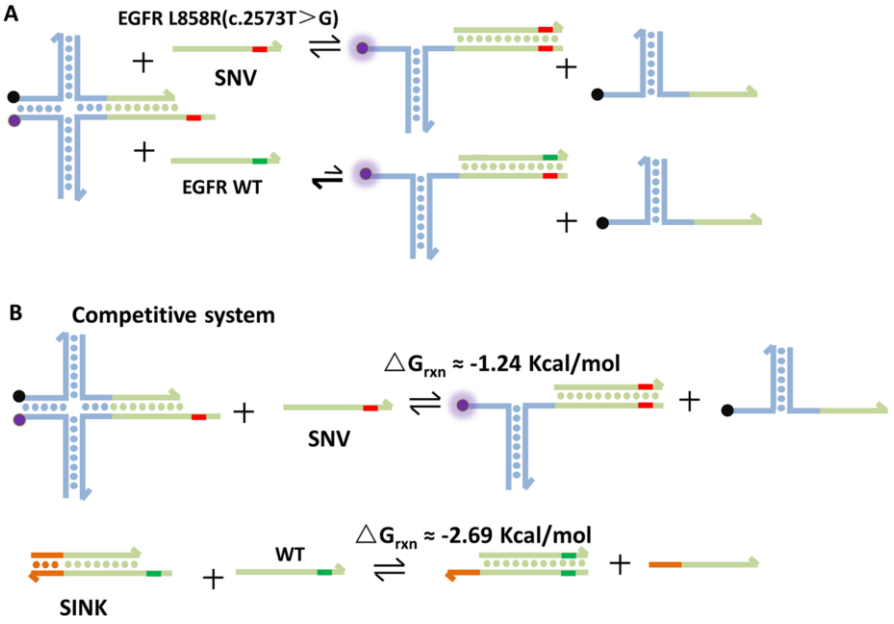
Variant allele frequency (VAF) is defined as the VAF $\cong \frac{[T]_0}{[N]_0}$.

This equation also takes into account the influence of the background, which is a constant, representing a collection of all sources of unavoidable detection signals (e.g., detector dark current, autofluorescence, ambient noise, etc.). It is worth noting that when the background is equal to 0, β is the same as DF, so β explains the specificity. When the background is positive, the minimum signal required for the detection mechanism increases, resulting in an increase in detection limit, a decrease in sensitivity, and a decrease in β value. Therefore, the β value also explains the sensitivity. Since it can explain the specificity and the sensitivity, the β value has a more accurate and comprehensive evaluation index than the DF value in the detection environment of the low abundance target.

As shown in Fig. 15, Zhang et al. [66] used X-probes to detect SNV. When VAF = 1%, the mutant nucleic acid EGFR L858R was low in abundance. In this case, the background signal is not negligible. In this system, $\beta = 145$ when VAF = 1%. Further, they proposed using a competitor to detect SNV to improve the specificity of the detection method. When VAF = 1%, $\beta = 560$, when VAF = 0.1%, $\beta = 2420$; when VAF = 0.01%, $\beta = 5460$. In the lower VAF experiments, the β value is higher because of the relatively small contribution of background fluorescence. In addition, the β value takes into account the non-target signal interference under low abundance conditions, and can more accurately judge the specificity of the method.

4 Conclusion and Prospects

Techniques that can detect SNVs with high specificity and high sensitivity are of great value for both fundamental research and clinical applications. Recent advances in DNA strand displacement provide ever finer tools to develop sensitive methods for SNV detection. In this review, we highlighted different strand displacement techniques that are universal and can be generalized to most nucleic acid targets of interest. Therefore, they hold great application potential. These techniques generally rely on the specificity of Waston–Crick complementary base pairing, and each strategy has its own advantages and disadvantages. For SNV detection based on toehold-mediated strand displacement reaction, the discrimination factor based on the initial data response rate detection mode is much higher than that based on the maximum strength detection mode. Moreover, the method is sensitive only to mutations occurring in and near the toehold region, and is insensitive to SNVs that are far away from the toehold. For SNV detection based on toehold exchange, it can specifically identify SNV in any region but with the disadvantage of low hybridization yield and long reaction time. The DNA polymerase-mediated primer extension reaction is involved in recognition of SNV as an amplification component or as a sensitive



C

X Probe system	
VAF	β
1%	145
X Probe with SINK system	
VAF	β
1%	560
0.1%	2420
0.01%	5469

Fig. 15 **a** SNV detection based on X probe. **b** Competitive composition here consists of a target-specific X-Probe and a WT-specific Sink, with near-optimal ΔG° rxn values (kcal mol^{-1}). **c** Different variant allele frequencies (VAF) of the target [66]

detection method for the 3′-end mutation, supplementing the defect of terminal mutation detection.

To further expand the ultrasensitive SNV detection techniques for actual samples, several challenges remain to be addressed. First, it is known that mutant nucleic acids in an actual sample are low-abundance, and samples usually contains a large number of highly homologous sequences. Thus, methods with high selectivity and high specificity are desirable. Priority enrichment methods have been developed to increase the collision probability between the probe and the target to increase specificity. Or, through competitive separation strategies, unexpected reactions in the mixture can be eliminated, ensuring probe selection and improved recognition specificity. These methods are designed to ensure the probe prefers to react with target, thereby increasing specificity and selectivity. Second, many analytical methods

require the design of an equal number of probes to identify multiple different SNVs, which results in unnecessary manual checks and increased system complexity. Therefore, DNA logic circuits are very promising in the application of multiple SNVs identification. Third, the combination of multiple strategies is a good solution when conventional detection methods fail to provide the required performance. For example, more SNV recognition strategies in series can obtain high specificity. The combination of recognition strategy and amplification strategy can address the trade-offs of specificity and sensitivity. Towards these challenges, we anticipate that the unique properties of DNA strand displacement techniques will be continuously explored, generating powerful analytical tools for nucleic acid analysis and clinical diagnosis in the near future.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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