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A two-layer assay for single-nucleotide variants utilizing strand displacement and selective digestion

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ABSTRACT

Point mutations have emerged as prominent biomarkers for disease diagnosis, particularly in the case of cancer. Discovering single-nucleotide variants (SNVs) is also of great importance for the identification of single-nucleotide polymorphisms within the population. The competing requirements of thermodynamic stability and specificity in conventional nucleic acid hybridization probes make it challenging to achieve highly precise detection of point mutants. Here, we present a fluorescence-based assay for low-abundance mutation detection based on toehold-mediated strand displacement and nuclease-mediated strand digestion that enables highly precise detection of point mutations. We demonstrate that this combined assay provides 50–1000-fold discrimination (mean value: 255) between all possible single-nucleotide mutations and their corresponding wild-type sequence for a model DNA target. Using experiments and kinetic modeling, we investigate probe properties that obtain additive benefits from both strand displacement and nucleolytic digestion, thus providing guidance for the design of enzyme-mediated nucleic acid assays in the future.

1. Introduction

Precise nucleic acid recognition is of great importance for the development of molecularly precise, individualized medicine (Bianchi, 2012; Topol, 2014). Low-frequency mutations have attracted increasing attention as a specific biomarkers for disease diagnosis and prognosis (Loeb, 2011; Vasan, 2006). Hence, much effort has been made to enhance the capability of nucleic acid assays to discriminate among SNVs, including such approaches as specific PCR (Taly et al., 2013), barcode-based assays and next-generation sequencing (Schmitt et al., 2012). Hybridization probes (Knez et al., 2014) such as molecular beacons, binary probes and artificial nucleotide probes provide an effective way to detect mutations for which the sequences of the corresponding wild type and the mutant are known. However, the poor specificity of nucleic acid hybridization at room temperature-typically far below the melting temperature of probe-target duplexes-hampers its capacity to detect low-frequency point mutants (SantaLucia and Hicks, 2004).

The differential activity of some nucleases towards fully matched and partly mismatched double-stranded DNA (dsDNA) has been employed to enhance the specificity of some hybridization-based assays (Wu et al., 2015; Xiao et al., 2013). However, nuclease-assisted nucleic acid assays usually show strong sequence dependence, with poor specificity (<5-fold) towards some types of point mutations, restricting their widespread application in genotyping.

Meanwhile, the development of strand exchange and displacement reactions in dynamic DNA nanotechnology has resulted in an unprecedented level of control over hybridization reactions. Toehold exchange (TE) permits rapid equilibration between competing dsDNA species through the introduction of short overhang sequences that facilitate strand exchange. The kinetics and thermodynamics of this process can be highly sensitive to even single-base mismatches (Machinek et al., 2014), allowing for SNV detection (Khodakov et al., 2015). Taking advantage of these features of TE, Zhang et al. (2012) optimized the specificity of nucleic acid detection, offering typically 20–50-fold discrimination for a point mutants. More recently, they showed that the discriminating power of TE can be greatly improved through the simulation-guided design of probe-competitor systems (Wang and Zhang, 2015).

Herein, we demonstrate a kinetic method for the sensitive detection of low-abundance mutations against a large background of wild type DNA by controlling the kinetics of TE and lambda exonuclease digestion (LED) simultaneously. Conceptually, the TE

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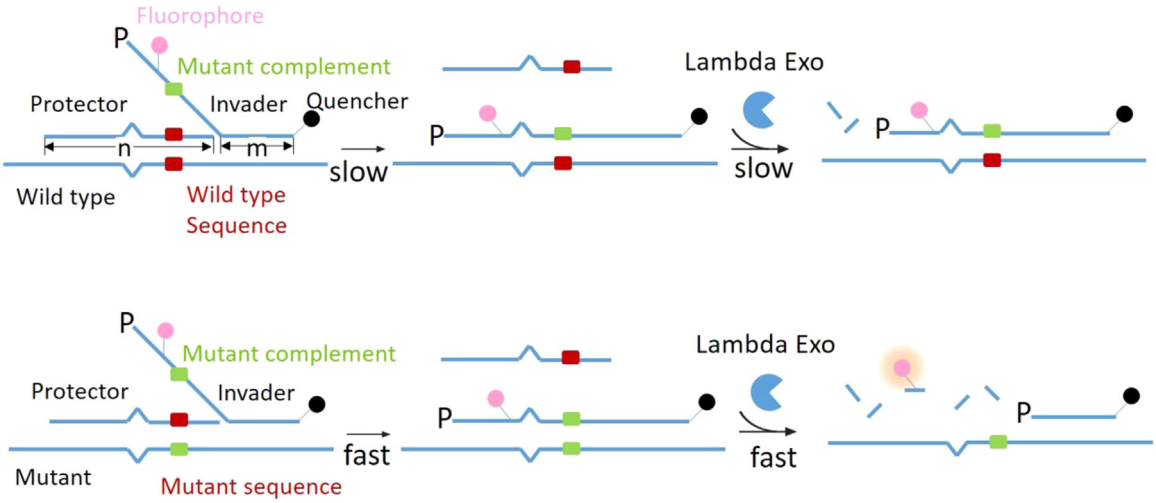

Fig. 1. Schematic illustration of toehold exchange nuclease-assisted single-nucleotide discrimination (TEND). The internal loop represents the purposefully introduced mismatch to improve discrimination by lambda exo. P=5′-phosphate modification.

provides a highly mutation-sensitive gating mechanism, while the LED provides additional discrimination as well as an irreversible, signal-generating step. Lambda exonuclease (lambda exo) catalyzes the stepwise hydrolysis of the 5′-phosphorylated strand of a DNA duplex in the 5′ to 3′ direction (Lee et al., 2011). In the assay, we introduce a protector probe which forms one mismatch with the wild type strand but two mismatches with the mutant strand (Fig. 1). Upon removal of the protector strand by TE with an invader strand, LED of the 5′-phosphorylated invader strand (now in dsDNA form) can proceed. The mismatches serve two functions: first, as described above, the kinetics of TE is sensitive to the number and position of mismatched bases in a duplex, permitting TE to discriminate between the wild-type and mutant sequences. Second, the mismatch common to both wild type and mutant (internal loop, Fig. 1) is introduced to improve discrimination by LED. As reported previously, lambda exo can discriminate between doubly and singly mismatched duplexes, but not between a singly mismatched and a fully complementary duplex (Wu et al., 2015). Thus, the additional mismatch ensures that the invader bound to the mutant sequence will be digested much more rapidly than invader bound to the wild type sequence. For signal generation by dequenching of fluorescence upon strand digestion, the invader strand was labeled with a fluorophore internally and with a quencher at the 3′ end. The assay was performed by monitoring the rate of fluorescence increase in a one-pot reaction comprising both TE and LED steps. We term this combined system toehold exchange nuclease-assisted single nucleotide discrimination (TEND).

2. Materials and methods

2.1. Materials

Lambda exonuclease, Exonuclease I (Exo I), Taq DNA polymerase and their corresponding buffers were obtained from New England Biolabs (MA, USA). All of the oligonucleotides used in this work were synthesized and purified by HPLC (Sangon Co., China) and their sequences are listed in Table S1. DNase/RNase free deionized water from Tiangen Biotech Co. (Beijing, China) was used in all experiments.

2.2. Toehold exchange-nuclease mediated single nucleotide discrimination (TEND) assays of synthetic oligonucleotides

All reactions were carried out inside a sealed 200-μL PCR tube. Prior to adding invader and enzyme, the target and corresponding protector were mixed well in ThermoPol buffer (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton® X-100, pH 8.8@25 °C), heated to 85 °C, and then gradually cooled down to 37 °C to anneal the target-protector duplex. The labeled invader and enzyme were added simultaneously. In a 50 μL reaction system, the final concentrations of target, invader, and lambda exonuclease were 100 nM, 200 nM, and 16.7 units/ml, respectively. The protector concentration was varied from 0 to 800 nM as described in the main text. Fluorimetry was performed in the SYBR Green/FAM channel of a Rotor-Gene Q 5plex HRM Instrument (QIAGEN, Hilden, Germany) at 37 °C, using a gain of 8, a time interval of 5 s between reads, and an acquisition time of 10 min. The rate of fluorescence increase was determined by a linear regression fit to the initial linear portion of the time curve. In the optimization experiments, the concentrations of species, acquisition time and temperature varied but the same procedure was used.

2.3. Detection of single nucleotide variants at low frequency

Samples of single-stranded DNA bearing either the wild type or single-nucleotide mutant sequence were mixed at different ratios (10%, 1.0%, 0.5%, 0.2% and 0%); the total concentration was fixed at 100 nM. After adding protector strand to a final concentration of 200 nM and carrying out the same heat-annealing procedure described above, the invader and enzyme were added, and the fluorescence signal vs. time was recorded as described above.

2.4. Detection of KRAS codon 12 135G > A mutation at different frequencies

PCR was performed using *Taq* DNA polymerase (1.25 U) in ThermoPol Buffer in a 50 μL reaction volume containing 240 nM of primers, 0.2 mM dNTPs and 20 pM mixed synthetic template (wild type and mutant at different ratios). The PCR amplification regime (94 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s, 25 cycles) was used on a Rotor-Gene Q 5plex HRM Instrument. After the PCR amplification, exonuclease I (5 U) was added to remove the single strand primers, followed by inactivation at 85 °C for 10 min. Then lambda exo (5 U) was added to digest the 5′-phosphorylated strands (i.e., those extended from the forward primer only) within the duplex products for 20 min at 37 °C. After inactivation of lambda exo at 85 °C for 10 min, protector was added and mixed well, followed by the TEND assay as described above.