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Colorimetric detection of single nucleotide polymorphisms in the presence of 10^3-fold excess of a wild-type gene

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ABSTRACT

Herein, we proposed a simple colorimetric assay for highly sensitive and specific detection of single nucleotide polymorphisms (SNPs). Briefly, SNP specific capture probes (CPs) were immobilized onto magnetic beads. The hybridization of a target SNP with the CPs and detection probes containing multiple DNAzyme sequences (DNAzyme-DPs) brought the target SNP and the DNAzyme-DPs onto the magnetic beads. Meanwhile, a mismatch-specific CEL II enzyme (Surveyor® nuclease) cleaved the imperfectly hybridized wild-type gene together with all other mismatched sequences off the magnetic beads, leaving only the perfectly matched SNP strands on the magnetic beads. Amplified colorimetric detection was carried out through the DNAzyme-catalyzed oxidation of 3,3',5,5'-tetramethylbenzidine in the presence of H_2O_2. The excellent selectivity of Surveyor® nuclease toward all imperfectly-matched DNA duplexes produced an ultrahigh selectivity – one mutant in 1000 copies of the wild-type gene can be detected. In addition, the cumulative nature of the DNAzyme-amplified signal generation process produced a detection limit as low as 0.40 fM and a dynamic range from 1.0 to 200 fM. The simple protocol and its high sensitivity and selectivity allowed the proposed assay to be used in the detection of SNPs in genomic DNA samples.

1. Introduction

The importance of SNPs, especially on the genetic diseases, has urged researchers to develop SNP detection methods for their sensitive and accurate identification in the presence of excessive wild-type (WT) genes. Unlike other DNA assays, the intrinsically subtle difference between WT and mutant genes – a single base variation – makes it a challenging task to specifically detect low abundant SNPs out of large amounts of co-existing WT genes. To date, many methods have been proposed for SNP detection and they can be classified into two categories – allele-specific hybridization methods and allele-specific enzymatic methods (Syvänen, 2001). The allele-specific hybridization methods lie in the detection of the subtle difference of stabilities caused by the single base mismatch (Wallace et al., 1979; Kennedy et al., 2003; Xu et al., 2003; Mir and Southern, 1999). However, the thermodynamically driven hybridization suffers from the problems of low sensitivity and specificity (Teo, et al., 2014). On the other hand, the allele-specific enzymatic methods, such as primer extension (Kim and Misra, 2007), enzymatic ligation (Landegren et al., 1988; Shen et al., 2013a), and enzymatic cleavage (Myakishev et al., 2001; Chen et al., 1998; Nollau and Wagener, 1997), are much more specific. To substantially enhance the sensitivity, the enzyme-based SNP assays are usually coupled to various amplification methods (Duan et al., 2010), such as polymerase chain reaction (PCR), ligase chain reaction (LCR) (Wee et al., 2012; Shen et al., 2012), and rolling circle amplification (RCA) (Lizardi et al., 1998). For example, Wee and co-workers (Wee et al., 2012) proposed a highly sensitive SNP assay by combining LCR with enzymatic signal amplification, a sub-picomolar detection limit, and a selectivity factor of 1000 were achieved. More recently, a nanogap-based electronic sensor array along with nucleases was developed for highly selective and label-free detection of SNP (Shen et al., 2013b). Although good performance was obtained, the involvement of electron beam lithography in the fabrication of the sensor array may hinder its applications at point-of-care.

Intensive research activities in SNPs have prompted the development of simple, robust, highly sensitive and specific SNP assays for uses at point-of-care. In spite of the excellent sensitivity and specificity of allele-specific enzymatic methods, the requirements for special laboratory skills in sample preparation and for complex equipment in signal readout or/and thermal cycler for amplification severely hinder their adaption to decentralized settings like point-of-care and field use. In contrast, colorimetric detection would significantly reduce the cost and complexity of

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signal readout, allowing SNP detection to be performed with high confidence at point-of-care. Unfortunately, most of the reported colorimetric SNP assays are not sensitive or selective enough to be viable alternatives (Li et al., 2010; Chen et al., 2012). In an effort to significantly enhance the sensitivity and selectivity of colorimetric SNP assays without engaging any thermal application techniques and greatly simplify the assay protocol, herein we proposed a colorimetric assay for highly sensitive and selective detection of SNPs. The assay coupled the high specificity of a mismatch-specific CEL II enzyme (Surveyor® nuclease) in cleaving mismatched (interfering) DNA duplexes to the excellent signal amplification power of DNAzyme and the simplicity of utilizing magnetic beads in the separation of hybridized target SNPs. Consequently, in addition to its simple protocol and signal readout mode, excellent sensitivity and selectivity were observed. Unlike the thermal amplification strategies employed in the allelic specific enzymatic techniques such as LCR and PCR, the isothermal protocol employed for both Surveyor® nuclease cleavage and DNAzyme amplification is advantageous in the development of a robust SNP detection platform, which may bring SNP detection one step closer to point-of-care.

2. Experimental section

2.1. Materials and apparatus

Surveyor® nuclease kit was purchased from Transgenomic, Inc (Omaha, NE, USA). Streptavidin-coated magnetic beads (2.8 μm, M-280 Dynabeads, monodispersed with $<5\%$ batch-to-batch variations) were acquired from Life Technologies (Carlsbad, CA) and their transmission electron microscopic (TEM) images were shown in Fig. 1. Three types of biotinylated capture probe (CPs), DNAzyme-terminated detection probes (DNAzyme-DPs), synthetic genes (KRAS oncogenes of WT and mutants) and all other synthetic DNAs were custom-made by Integrated DNA Technologies (Coralville, IA). Sequence information of KRAS oncogenes is as follows: AAA CTT GTG GTA GTT GGA GCT – mutation site (G: WT, A: A-mutant, T: T-mutant, C: C-mutant) – GT GGC GTA GGC AAG AGT GCC TTG. Hemin ($>98\%$) and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich Co (St Louis, MO). All other chemicals of certified analytical grade were from Sigma-Aldrich and used as received. Nuclease-free water was used for all aqueous solution preparations. CP binding buffer consisted of 5.0 mM Tris–HCl, 0.5 mM EDTA, and 1.0 M NaCl. Hybridization buffer was made of pH 8.0 20 mM Tris–HCl, 1.0 mM EDTA, 25 mM $MgCl_2$, and 0.10 M NaCl. Colorimetric measurements were carried out using microcuvettes on an Agilent Cary 60 UV–visible spectrophotometer (Agilent Technologies, Inc. Santa Clara, CA).

2.2. Conjugation of the CPs to the magnetic beads

The conjugation of the biotinylated CPs to the streptavidin-coated magnetic beads was performed according to the recommended procedure by Life Technologies with slight modification. Briefly, 10 mg of the streptavidin-coated magnetic beads was washed three times with the binding buffer with the help of a permanent magnet and resuspended in 1.0 mL of the binding buffer. Then, the biotinylated CPs were added into the above solution with a final concentration of 5.0 μM, mixed well by vortexing, and the reaction mixture was incubated for 20 min at room temperature with gentle vortexing to ensure the completion of the interaction between biotin and streptavidin. The concentration of the CPs in the supernatant was then analyzed. It was estimated that the average surface coverage is 4.3×10^5 strands/magnetic bead, representing ~20% of the binding capacity of the magnetic beads (Life Technologies, 2011). Finally, the CP-conjugated magnetic beads were separated from the reaction mixture, carefully washed with the hybridization buffer, and resuspended in 1.0 mL of the hybridization buffer. The CP-functionalized magnetic beads were kept at 4 °C and ready for use.

2.3. SNP detection

After a short heat-treatment at 95 °C for 5 min a 20-μL aliquot of a mutated gene (SNP) was first incubated in the hybridization buffer containing 5.0 U Surveyor® nuclease, 1.0 μM DNAzyme-DPs, and 100 nM CP-coated magnetic beads at room temperature for 60 min to capture the mutated gene and the DNAzyme-DPs. Maximal hybridization efficiency was attained because of the low hybridization stringency. Moreover, interfering sequences (WT gene and imperfectly matched genes) were also captured during hybridization because of the low hybridization stringency, but they were instantaneously cleaved off by Surveyor® nuclease. Thereafter, the magnetic beads along with the captured SNP and DNAzyme-DPs were separated from the hybridization buffer. After three thorough washes with the hybridization buffer, the magnetic beads were re-suspended in pH 4.0 2.0 mM TMB+25 mM H_2O_2 in methanol/water (1/2) mixture, briefly vortexed, and incubated for 30 min at room temperature in the dark with gentle vortexing. Finally, the absorbance was measured at 370 nm. Longer periods of hybridization and TMB incubation allowed an improved detection of SNPs, while a period of 60 min hybridization was found to be sufficient to quantify SNPs down to femtomolars, comparable

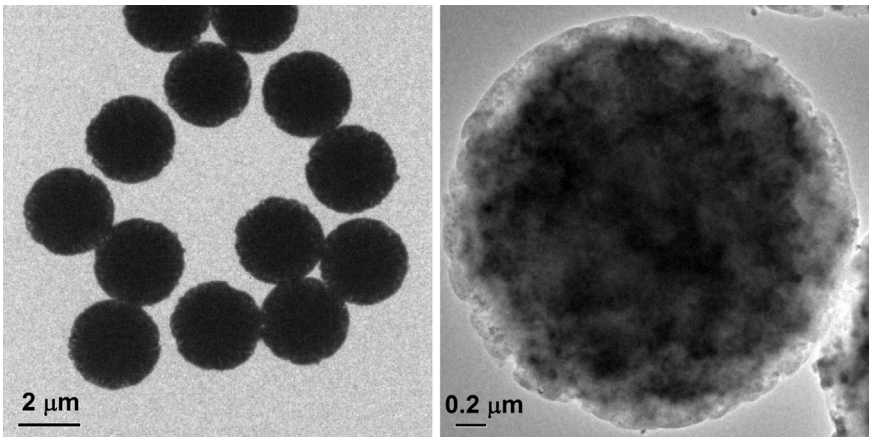

Fig. 1. TEM images of the 2.8-μm magnetic beads.