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Homogeneous and one-step fluorescent allele-specific PCR for SNP genotyping assays using conjugated polyelectrolytes

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ABSTRACT

A new label-free, homogenous, sensitive and economical one-step method to detect SNP genotyping of genomic DNA has been developed by combining allele-specific PCR technique with water-soluble cationic conjugated polyelectrolytes (CCP). The amplification of target DNA and fluorescence detection steps are combined into one-step. The target DNA fragment containing a G allele site acts as PCR template. For the G allele-specific forward primer whose 3′-terminal base is complementary to the G allele template, after the first step of reverse primer extension, G allele-specific primer perfectly anneals with newly formed strand and the extension reaction of forward primer starts. During the extension, dGTP-Fl and dUTP-Fl are incorporated into extension chain in the presence of Taq DNA polymerase and more fluorescein-labeled PCR amplicons are yielded. Upon adding the CCP, strong electrostatic interactions between DNA and CCP bring them close and efficient FRET from CCP to fluorescein occurs. For the C allele-specific forward primer, less fluorescein-labeled PCR amplicons are yielded and inefficient FRET occurs. By triggering the change of emission intensity of CCP and fluorescein, it is possible to assay the SNP genotypes. In contrast to previous reports, this method does not require designing dye-labeled primers, and gel electrophoresis and isolation step after PCR were avoided in this homogenous method. The genotyping of 50 ng genomic DNA from human lung cancer cell is easily detected using our new method. These qualities will make the new detection system ideal for SNP genotyping.

1. Introduction

Single nucleotide polymorphisms (SNPs) are the most common type of sequence variants in human genome (Kruglyak and Nickerson, 2001). Inherited genetic variation might have an important role in the pathogenesis of disease. Developing a rapid, cost-efficient, sensitive method for SNP genotyping would benefit the prevention, diagnosis and treatment of disease (The International HapMap Consortium, 2003, 2005; Crawford and Nickerson, 2005). Various methods for SNP genotyping detection have been reported (Kwok, 2001; Kim and Misra, 2007), such as single strand conformation polymorphism analysis, heteroduplex analysis, allele-specific hybridization, enzyme mismatch cleavage and ligation assays. In these methods, two-steps are required: the first one is the amplification of the target DNA fragment from the genome by PCR; the second one is the detection of SNP genotypes in amplification products. Most of these detections are based on electrophoresis separation or heterogeneous assay formats, which are time- and labor-intensive because of the multiple separation or wash steps. Although homogeneous assays have been developed, such as fluorescence polarization and invader assays, they either require expensive duel-labeled probes or complex detection procedure (Tyagi and Kramer, 1996). The rapid and economical methods combining the amplification and detection steps into one-step have been developed for SNP genotyping, such as homogeneous allele-specific PCR with fluorescence detection based on molecular beacon, TaqMan or DNA enzyme (Kwok, 2001; Myakishev et al., 2001; Whitcombe et al., 1998). However, these methods still require expensive duel-labeled probes or complex instrument.

Water-soluble conjugated polyelectrolytes (CPs) contain a large number of absorbing units, and the transfer of excitation energy along the whole backbone of the CP to the chromophore reporter results in the amplification of fluorescence signals (Thomas et al., 2007; Liu and Bazan, 2004; Chen et al., 1999). Therefore, CPs can be used as the optical platforms in highly sensitive chemical and biological sensors (Ho et al., 2005, 2008; Yang et al., 2005; Gaylord et al., 2002; Xu et al., 2005; Feng et al., 2007; He et al., 2005; Lee et al., 2007; Li et al., 2005; Nilsson and Inganäs, 2003). More recently, we have been developed a two-step SNP genotyping method based on cationic CPs and allele-specific primer extension

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(Duan et al., 2007, 2008). In these works, two-steps are required: the first one is the amplification of the target DNA fragment; the second one is the single base extension reaction followed by the detection of SNP genotypes using CPs. Furthermore, the target ssDNA should be from the PCR product that was amplified from genomic DNA, and the genomic DNA could not be detected directly. Here we present a new homogenous, sensitive and economical one-step method to detect SNP genotype in genomic DNA taking advantages of allele-specific PCR technique with light-harvesting property of CPs. The present work is a one-step method for SNP genotype detection and only needs the amplification of target DNA by PCR followed by the detection using CPs, thus the genomic DNA could be detected directly. Use genomic DNA as start material is more simply and efficient, which is important because of the large quantity of the SNPs. Compared to those established homogeneous allele-specific PCR systems, our new assay method has two important features: (1) through the strong electrostatic interaction water-soluble cationic CPs and negatively charged DNA can form a complex, it does not require fluorescent labels on the primers, which should significantly reduce the cost; (2) the amplification of fluorescence signals from CPs and the ratiometric fluorescence measurement guarantee an easier detection of low-concentration analyte contrast to the background, which improves the detection sensitivity.

2. Experimental methods

2.1. Materials and measurements

PAGE-purified oligonucleotides were purchased from SBS Genetech Co., Ltd. HotMaster Taq DNA polymerase was obtained from Tiangen Biotech (Beijing) Co., Ltd. Shrimp alkaline phosphatase (SAP) was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The dGTP-Fl was purchased from Perkin Elmer. The dUTP-Fl was obtained from Fermentas. SYBR gold was obtained from Invitrogen. The genomic DNA was obtained from human lung cancer cell A549. The synthesis of PFP is described in the literature (Stork et al., 2002). All the PCR reactions were carried out in a Bioer Little Genius TC-25/H Thermal Cycler. A Hitachi F-4500 spectrofluorimeter equipped with a 150 W high-pressure Xenon lamp was used to obtain the fluorescence spectra. DYY-6C electrophoresis power supply and DYCZ-24D polyacrylamide gel electrophoresis cell (Beijing Liuyi Instrument Factory) were used in polyacrylamide gel electrophoresis (PAGE) analysis. The image was recorded by a Canon IXUS 750 digital camera in a WD-9403F UV Viewing Cabinet (Beijing Liuyi instrument factory, Beijing) equipped with a 550–650 nm band-pass filter.

2.2. The allele-specific PCR

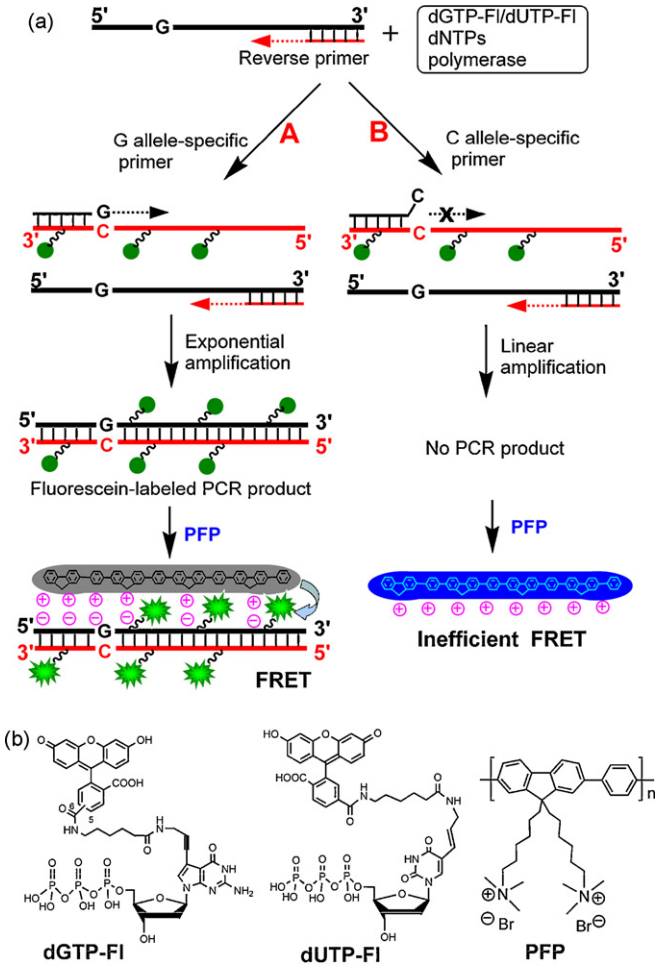
The PCR mixture contained 1× HotMaster Taq DNA polymerase PCR buffer, 0.5 μM G allele-specific primer or C allele-specific primer and reverse primer, 10 μM of dATP, dCTP, 5 μM of dUTP-Fl, dGTP-Fl, dTTP and dGTP, 0.5 unit HotMaster Taq DNA polymerase, and 2 μL template DNA (1 pM) in a total volume of 20 μL solution. After 95 °C for 3 min, the thermocycling parameters were as follows: 30 cycles at 94 °C for 30 s and 60 °C for 1 min. After the cycle reaction, the extension reaction was carried out for 5 min at 65 °C, and then the reaction products were held at 4 °C. For the PCR reaction of genomic DNA, a solution of containing 1× HotMaster Taq DNA polymerase PCR buffer, 0.5 μM G allele-specific primer or C allele-specific primer and reverse primer, 10 μM of dATP, dCTP, 5 μM of dUTP-Fl, dGTP-Fl, dTTP, dGTP, 1 unit HotMaster Taq DNA polymerase and 50 ng A549 genomic DNA was established. After 94 °C for 5 min, the thermocycling parameters were as follows: 35 cycles of 94 °C for 15 s, 60 °C for 20 s and 65 °C for 40 s. After the cycle reaction, the extension reaction was carried out for 5 min at 65 °C, and then the reaction products were held at 4 °C.

2.3. Electrophoresis analysis

PCR products were loaded onto 15% non-denaturing polyacrylamide gel in 1× TBE buffer. The electrophoresis was performed at 12 V/cm until the bromophenol blue dye band in the marker lane migrated approximately 2.5 cm. After electrophoresis, the gel was stained by 1× SYBR gold in 1× TBE buffer for 30 min. The photograph of gel was taken by a digital camera in UV viewing cabinet equipped with a 550–650 nm band-pass filter.

2.4. Fluorescent measurement

Before fluorescent measurement, 4 μL shrimp alkaline phosphatase (0.5 unit/μL) was added into PCR products and was incubated at 37 °C for 20 min to degrade unreacted dNTP-Fl. After



Scheme 1. (a) Schematic representation of the SNP genotyping assay. (b) Chemical structures of PFP, dGTP-Fl and dUTP-Fl and DNA sequences used in the assay.