Integration of rolling circle amplification and cationic conjugated polymer for the homogeneous detection of single nucleotide polymorphisms

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A novel, homogeneous and sensitive assay for the detection of single nucleotide polymorphisms (SNPs) by integration of rolling circle amplification (RCA) and cationic conjugated polymer (CCP) has been developed and tested. Mutant DNA serves as the template for specifically circularizing a padlock probe (PLP) with a sequence that is complementary to the mutant DNA. Afterwards, the mutant DNA directly acts as the primer to initiate the RCA reaction in the presence of phi29 DNA polymerase that generates a long, tandem single-strand DNA product. During the RCA reaction, fluorescein-labeled dUTPs are incorporated into the RCA products. When the CCP is introduced, efficient FRET from CCP to fluorescein occurs as a result of the strong electrostatic interactions between the CCP and the DNA produced by RCA. The wild-type DNA contains a single base mismatch with PLP with the result that the PLP is not circularized, RCA is not triggered and inefficient FRET results. By measuring the change of the emission intensities of CCP and fluorescein, it was possible to detect the SNP in a homogeneous manner. The method is sensitive and specific enough to detect 0.1 pmol/L mutant DNA and to determine a mutant allele frequency as low as 2.0%.

rolling circle amplification, cationic conjugated polymer, single nucleotide polymorphisms, homogeneous detection

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Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variation. They have been widely used as efficacious genetic markers for comprehensive genetic analyses of complex diseases and drug responses in genome-wide association studies [1]. The detection of SNPs paves the way for the diagnosis and treatment of diseases [2]. Various heterogeneous and homogeneous methods for SNP detection have been reported. The heterogeneous formats for SNP detection generally require separation [3] or immobilization of the capture probe on a solid support phase such as a chip [4], a magnetic bead [5] or a nanoparticle [6]. This results in multiplex steps, high costs and longer analytical times. To detect homogeneous SNPs, a molecular beacon [7] or a TaqMan probe [8] and fluores-

cence polarization [9] is usually used. These require expensive dual-labeled fluorescent probes or complex detection procedures. Pyrophosphate detection-based homogeneous SNP assays require stepwise chemical reactions with multiple enzymes resulting in complex steps and high costs [10,11].

Cationic conjugated polymers (CCPs) with a large number of repeated absorbing units can transfer the excitation energy along the whole CCP backbone to the chromophore reporter [12]. Because of their unique light-harvesting properties, CCPs provide an effective platform for homogeneous SNP detection based on the formation of polyelectrolyte complexes stabilized by electrostatic interactions between the CCPs and negatively charged DNA. When a fluorophore-labeled nucleotide is incorporated into the DNA chain during the primer extension reaction at an SNP

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site, higher fluorescence resonance energy transfer (FRET) efficiency between the CCP and the fluorophore-label nucleotide is observed. By monitoring the ratio of fluorescent emission intensity of the CCP and the fluorophore, homogeneous SNP detection can be achieved [13,14]. However, because a single-base extension reaction is used, this method suffers from lack of sensitivity. To improve the sensitivity of the method for SNP detection, Duan et al. [15] combined allele-specific PCR with CCPs and developed a multiple base extensions reaction using fluorescein-labeled dGTP and dUTP simultaneously. The further development of this simple, sensitive and cost-effective method is desirable.

Rolling circle amplification (RCA) is another versatile amplification technique in which a circularized padlock probe (PLP) is used as the template to create a long ssDNA product that contains several hundred tandem repeats of the complementary sequence to the PLP [16,17]. In the RCA process, ssDNA or a target sequence is used as the primer which is extended by DNA polymerases with strongly DNA strand displacement properties resulting in base extensions in the tens of thousands. Compared to the polymerase chain reaction (PCR), RCA exhibits several distinct advantages such as high specific ligation of PLP, isothermal amplification without high-precision temperature cycling and linear kinetics. Because it is simple and robust, RCA can be used to directly generate densely labeled amplification products with fluorescently labeled deoxynucleotide triphosphates (dNTPs) [18]. However, this method is often performed using surface-immobilized RCA that required complex separation and washing procedures.

In this paper, we present a simple and reliable homogeneous SNP detection method that integrates RCA and CCP and improves the sensitivity of the SNP assay. The target DNA is amplified by RCA and fluorescein-labeled dUTPs are incorporated into the DNA sequence during the RCA process. When the CCP is added, because of the strong electrostatic interactions between CCP and DNA, effective FRET from the CCP to the fluorescein DNA can be observed. This novel method is homogeneous, isothermal, and does not require specific instrumentation or high-precision temperature cycling.

1 Materials and methods

1.1 Materials

The synthetic DNA used in this study was obtained from

TaKaRa Biotechnology Co., Ltd. (Dalian, China). The sequences of the probe and targets are listed in Table 1. The mutant-type DNA (mutDNA) and wild-type DNA (wtDNA) are fragments of the human *p53* gene (exon8). All the oligonucleotides were PAGE purified. Ampligase Thermostable DNA Ligase and Phi29 DNA Polymerase were purchased from Epicentre Technologies (Madison, WI, USA). The deoxynucleotide triphosphates, fluorescein-12-dUTP (Fl-dUTP) and FastAP alkaline phosphatase (FastAP) were purchased from Fermentas China (Shenzhen, China). The cationic poly [(9,9-bis (6'-N,N,N-trimethylammonium)hexyl) fluorenylene-phenylene dibromide] (PFP) used as the CCP in the FRET experiments was synthesized according to the procedure reported by Stork et al. [19]. All solutions were prepared in deionized and sterilized water. The other reagents were of analytical reagent grade and used as purchased without further purification.

The 18 bases at the 5' (dotted) and 3' (underlined) ends of PLP are complementary to the dotted and underlined bases of mutDNA, respectively. At the polymorphic site, the C (underlined and in boldface) in the wtDNA is replaced by a T (underlined and in boldface) in the mutDNA. Abbreviations: mutDNA, mutant-type DNA; wtDNA, wild-type DNA; P, a phosphate group.

1.2 Ligation of padlock probe

For the ligation reactions, in each tube, 1 unit of Ampligase was added to 10 μL reaction mixture containing 20 mmol/L Tris-HCl (pH 8.3), 25 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L nicotinamide adenine dinucleotide (NAD), 0.01% Triton X-100, 50 nmol/L PLP and the appropriate amount of target DNA. After the initial incubation at 95°C for 3 min to denature the target DNA and padlock probe, 5 incubation cycles of 95°C for 1 min and 45°C for 5 min were followed by 45°C for 45 min.

1.3 RCA reaction

The product of the ligation reaction was added to 10 μL of RCA reaction mixture containing 80 mmol/L Tris-HCl (pH 7.5), 100 mmol/L KCl, 20 mmol/L MgCl₂, 10 mmol/L (NH₄)₂SO₄, 8 mmol/L dithiothreitol (DTT), 10 μmol/L of dATP, dCTP, dGTP, 9 μmol/L of dTTP, 1 μmol/L of Fl-dUTP, and 20 unit phi29 DNA polymerase. RCA reactions were performed at 37°C for 4 h and terminated by incubation at 65°C for 10 min.

Table 1 Sequences of the probe and targets

Probe	Sequence
PLP	5'-P-GTCTCTCCAGGACAGGCTTTTCATTTACAGTTAGCATTTGCGCATACTTCGGCTCTCTCTGTGCGCCA-3'
wtDNA	5'-GCGTGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAGAG-3'
mutDNA	5'-GCGTGTTTGTGCCTGTCCTGGGAGAGACTGGCGCACAGAGGAAGAG-3'

1.4 FRET detection of target DNA

Before measuring fluorescence, 1 μL FastAP (1 unit/ μL) was added to the RCA products and the mixture was incubated at 37°C for 20 min to degrade unreacted FI-dUTP. Aliquots of 6 μL RCA product and 6 μL PFP (20 $\mu\text{mol/L}$) were then added to a 1.5 mL centrifuge tube and diluted to 600 μL with 25 mmol/L HEPES buffer (pH 8.0). The fluorescence spectra were measured in a 1 cm $\times$ 1 cm quartz cuvette with a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) equipped with a Xenon lamp. The excitation wavelength was 380 nm, and the spectra were recorded between 400 and 600 nm.

1.5 Determination of allele frequencies

The mutDNA and wtDNA were mixed in ratios ranging from 2% to 100% and used as the DNA samples. The total amount of target DNA in each sample was 50 pmol/L. Fluorescence spectra of the synthetic DNA samples were obtained after ligation, RCA, and CCP-based FRET detection as described above.

2 Results and discussion

2.1 The principle of SNP detection

The new SNP detection method reported here is described schematically in Figure 1. Because the 18 bases at the 5' and 3' ends of the PLP sequence are perfectly complementary to the mutDNA, PLP is ligated and circularized in the presence of Ampligase. Subsequently, the mutDNA directly acts as a primer to amplify the circularized PLPs and initiate the RCA reaction which generates a long, tandem single-strand DNA product. In the presence of phi29 DNA polymerase, FI-dUTPs (chemical structure is shown in Figure 2(c)) are incorporated into the DNA strands produced by RCA. When PFP (chemical structure is shown in Figure 2(c)) is added, strong electrostatic interactions between the PFP and DNA bring PFP close to the fluorescein and

efficient FRET from PFP to fluorescein occurs. In contrast, one base at the 3' end of the PLP is mismatched with the equivalent wtDNA and so, because of the high specificity of Ampligase, PLP cannot be circularized. Thus, with the uncircularized PLP, only limited DNA extension occurs and less fluorescein-containing DNA is produced. As expected, when PFP is added inefficient FRET is observed. It is, therefore, possible to distinctively detect SNPs in a homogeneous manner by measuring changes in the emission intensity of CCP and fluorescein interactions.

Figure 2(a) shows the fluorescence spectra obtained when PFP was added to the RCA products of wtDNA and mutDNA. The unreacted FI-dUTP was degraded by FastAP alkaline phosphatase before the fluorescent measurement was recorded to further weaken electrostatic interactions between FI-dUTP and the PFP and to keep the fluorescein away from the PFP for suppressing the blank signal. For mutDNA, efficient FRET from PFP to fluorescein took place. The fluorescence emission of PFP at 426 nm was significantly quenched and the fluorescence emission of fluorescein at 530 nm was observed. In contrast, for wtDNA, the PFP fluorescence only decreased weakly and the fluorescence emission of fluorescein was almost the same as the blank, indicating that the FRET was very limited. FRET efficiency, defined as the ratio of fluorescence intensity at 530 nm to that at 426 nm ($I_{530\text{ nm}}/I_{426\text{ nm}}$), was used to evaluate the selectivity for SNP detection. The results demonstrate the high selectivity of this method in discriminating a one-base mismatch that can be attributed to the different amplification efficiencies of mutDNA and wtDNA. The mutDNA primes RCA with circularized PLP, and generates large amounts of long DNA incorporating many fluorescein molecules which results in efficient FRET. The wtDNA initiates a limited extension along with uncircularized PLP, which incorporates fewer fluorescein molecules and gives rise to inefficient FRET.

The RCA reaction primed by target DNA was verified by agarose gel electrophoresis. As shown in Figure 2(b), the long strand DNA products of the RCA reaction primed by mutDNA are too large to enter the agarose gel as can be

Figure 1 Schematic representation of homogeneous SNP detection by integration of RCA and CCP.

Figure 2 (a) Fluorescence spectra from HEPES buffer solutions containing the PFP and RCA products of mutDNA and wtDNA; (b) agarose gel electrophoresis (1.0%) of the RCA products without adding any other dye. The reaction mixtures were 50 nmol/L PLP, blank (lane 1), 10 nmol/L wtDNA (lane 2) and 10 nmol/L mutDNA (lane 3). M was the marker. The RCA reaction time was 4 h; (c) chemical structures of FI-dUTP and PFP.

seen in lane 3. In contrast, no RCA products of the blank and wtDNA were observed in lanes 1 and 2. The results are in accordance with those from fluorescent detection shown in Figure 2(a).

The concentration of FI-dUTP used in the RCA process has an important effect on subsequent SNP detection. Thus, the ratio of FI-dUTP to dTTP used in the RCA, where the sum of FI-dUTP and dTTP was 10 $\mu\text{mol/L}$, was optimized. We performed the RCA reaction with ratios of FI-dUTP to dTTP of 1:4, 1:9 and 1:19. As shown in Figure 3, the FRET efficiency for the blank, wtDNA and mutDNA gradually decreased as the ratio decreased, but the ratio of FRET efficiency for mutDNA to that of wtDNA increased as the ratio of FI-dUTP to dTTP decreased. However, the FRET efficiency for mutDNA for the ratio of FI-dUTP to dTTP of 1:19 is notably lower than for the ratio of 1:9. Thus, we used FI-dUTP and dTTP in the ratio of 1:9 in the present study.

Figure 3 Effect of the ratio of FI-dUTP to dTTP on FRET efficiency in the blank, wtDNA (20 pmol/L) and mutDNA (20 pmol/L). RCA products were diluted 100 times with HEPES buffer solution (25 mmol/L, pH 8.0) before the fluorescence measurement. The error bars show the error estimated from three replicate measurements.

2.2 Detection of SNP

Under the optimized experimental conditions, the relationship between FRET efficiency ($I_{530\text{ nm}}/I_{426\text{ nm}}$) for mutDNA and the concentration of mutDNA was investigated. As shown in Figure 4, the FRET efficiency for mutDNA increased remarkably with increasing mutDNA concentration over the range 0.1 to 50 pmol/L. In contrast, the FRET response for wtDNA was low and increased only marginally with increasing wtDNA concentrations. This result can be attributed to the limited extension of the wtDNA with the uncircularized PLP. The difference in the FRET response between mutDNA and wtDNA indicates that the present method has high specificity for SNP detection. Using this method 0.1 pmol/L mutDNA could be detected. This is 10000 times lower than the concentrations that can be detected using single-base extension-based FRET SNP assays (10 nmol/L) [13].

2.3 Allele frequency determination

Quantifying allele frequency in DNA pools is important in association studies between SNPs and susceptibility to diseases [4]. To determine a mutant allele frequency using the proposed method, mutDNA and wtDNA were mixed at ratios of 0, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0. The total concentration of DNA in the samples was fixed at 50 pmol/L. Figure 5 shows the relationship between allele frequency and FRET efficiency ($I_{530\text{ nm}}/I_{426\text{ nm}}$) for the RCA products of the test samples. As the ratio of mutDNA in the test samples increased, the FRET efficiency also increased. This result indicates that the proposed method can be successfully applied for SNP determination in pooled DNA samples.

Figure 4 Relationship between FRET efficiency and the concentration of the target DNA. The FRET efficiency is the net intensity produced by the target DNA, where the blank signal is subtracted from each value. The inset shows the enlargement of the graph for mutDNA between 0.1 and 10 pmol/L. The sequential detection was carried out as described in Section 1. The error bars show the error estimated from three replicate measurements.

Figure 5 Relationship between allele frequencies and FRET efficiency of samples of mixed mutDNA and wtDNA in various ratios. The total concentration of DNA in the samples was 50 pmol/L. The sequential detection was carried out as described in Section 1. The error bars show the error estimated from three replicate measurements.

3 Conclusion

In summary, we have developed a homogeneous SNP detection assay by combining RCA with CCP. No expensive instruments or complex steps are required for this assay. Furthermore, because the RCA product tends to be localized and contained within one continuous giant DNA molecule, it is suitable for imaging analysis [20]. Thus, our study provides an improved method for single molecule imaging based on homogeneous RCA assays.

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