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Ligation-rolling circle amplification combined with γ-cyclodextrin mediated stemless molecular beacon for sensitive and specific genotyping of single-nucleotide polymorphism

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ABSTRACT

A novel approach for highly sensitive and selective genotyping of single-nucleotide polymorphism (SNP) has been developed based on ligation-rolling circle amplification (L-RCA) and stemless molecular beacon. In this approach, two tailored DNA probes were involved. The stemless molecular beacon, formed through the inclusion interactions of γ-cyclodextrin (γ-CD) and bis-pyrene labeled DNA fragment, was served as signal probe. In the absence of mutant target, the two pyrene molecules were bound in the γ-CD cavity to form an excimer and showed a strong fluorescence at 475 nm. It was here named γ-CD-P-MB. The padlock DNA probe was designed as recognition probe. Upon the recognition of a point mutation DNA targets, the padlock probe was ligated to generate a circular template. An RCA amplification was then initiated using the circular template in the presence of *Phi*29 polymerase and dNTPs. The L-RCA products, containing repetitive sequence units, subsequently hybridized with the γ-CD-P-MB. This made pyrene molecules away from γ-CD cavity and caused a decrease of excimer fluorescence. As a proof-of-concept, SNP typing of β-thalassemia gene at position −28 was investigated using this approach. The detection limit of mutated target was determined to be 40 fM. In addition, DNA ligase offered high fidelity in distinguishing the mismatched bases at the ligation site, resulting in positive detection of mutant target even when the ratio of the wildtype to the mutant is 999:1. Given these attractive characteristics, the developed approach might provide a great genotyping platform for pathogenic diagnosis and genetic analysis.

1. Introduction

Single nucleotide polymorphisms (SNPs) are point mutations that constitute the most common genetic variation in the human genome, which accounts for about 90% of all genetic variations and occur approximately every 100–300 bases [1]. Due to its prevalence in the population, SNP can be served as not only an important high-resolution biomarker for mapping gene, defining population structure, gene association studies, but also a fundamental tool for disease diagnostics, pharmacogenetic analyses and drug discovery [2,3].

Over past decade, a wide variety of advanced technologies have been developed for SNP genotyping [4–6]. In principle, these approaches can be broadly categorized into two types: allele-specific hybridization and enzymatic allele identification [7]. Allele-specific hybridization methods generally utilize thermal stability, differences in hybridization efficiency, and local surroundings of double-stranded DNA hybrids to discriminate the hybridization difference between perfectly matched and single-nucleotide mismatched targets in a single-step [8]. These assays are straightforward and inexpensive, but most of them rely on differences in the free energy of probe/target binding to achieve SNP genotyping. Such differences are often small and can vary significantly on the basis of target sequence. Therefore, these genotyping methods require complex probe designs and the careful control of hybridization conditions such as buffer composition, washing stringency, and melting temperature, which limit their use at point-of-care settings [9,10]. By contrast, SNP genotyping assays based on the enzymatic allele

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discrimination, such as primer extension [11], ligation [12], or cleavage [13], have been developed to improve the fidelity [14]. By virtue of its high fidelity in single-base identification and applicability for any target of interest, ligation reaction based on DNA ligase is considered to be an ideal tool for specific SNP genotyping [15]. To achieve desired SNP discrimination sensitivity, this reaction usually combines with some subsequent amplification techniques [16,17]. Among these amplification techniques, a simple but powerful isothermal amplification technique, rolling circle amplification (RCA), was usually used to amplify a short DNA primer to generate long linear single-stranded DNA (ssDNA) molecules with many repeated sequences which are complementary to the circular DNA template [18]. Remarkably, because the circular template can be synthesized from a padlock probe, whose 5′- and 3′- ends can hybridize precisely onto the target and then be ligated by a DNA ligase, the circularization of the padlock probes by DNA ligase in ligation-rolling circle amplification (L-RCA) is so special that one-base mismatch can be specifically discriminated.

In L-RCA based assays, target quantification is achieved through the quantification of the L-RCA product. Molecular beacon (MB), a class of hairpin-shaped oligonucleotide fluorescence probes, has been widely applied for quantitative methodologies [19,20]. Even though the MB structure is the advantage for recognizing their targets with high specificity, it is still far from being functionally ideal. For example, high levels of stem sequence-dependent background signal and false positive signals result from endogenous nuclease degradation and nonspecific binding are commonly observed for these probes due to instability of the stem in a complex biological environment [21]. Despite some novel strategies to modulate the stem stability, such as replacements of the original DNA base pairs with nuclease-resistant nucleic acids (homo-DNA, locked nucleic acid, peptide nucleic acid, etc.), use of a metal-dependent stem based on metal-DNA base pairs, and design of triplex hairpin-shaped or G-quadruplex probes [22–30], many of the existing MB probes still have presented disadvantages in terms of the complexity of the synthesis process, costliness of materials, toxicity of the heavy metal ion, supersubtle optimization, etc. Thus, it is still highly important and desirable to develop low-cost, efficient and universal MB for biochemical sensing. Recently, a γ-CD-mediated dual-pyrene-labeled stemless molecular beacon (γ-CD-P-MB) was easy constructed by bounding the two pyrene molecules labeled DNA probe in the γ-CD hydrophobic cavity [31]. By introducing γ-CD amplification, the two pyrene molecules are housed within the γ-CD cavity to trigger remarkable excimer fluorescence enhancement. γ-CD/pyrene bounding interaction also allows tuning the stem stability of the probes, thereby achieving higher target-binding selectivity and sensitivity than conventionally structured DNA probes [32]. The γ-CD-P-MB avoids any variation of the stem's length and sequences, which does eliminate restrictions on probe design. In addition, the MegaStokes shifting (~130 nm) and long fluorescence lifetime (~40 ns) of pyrene excimer (λ_{ex}=344 nm, λ_{em}=475 nm) prevent interference background problems such as autofluorescence from biological environments, providing an opportunity for detection of target from complex biological media [33–35].

In the context of our long-term interests in searching for simple, sensitive and highly specific enzymatic SNP genotyping method, L-RCA technology and the stemless molecular beacon γ-CD-P-MB were integrated. In this protocol, the padlock probe was designed as recognition probe for mutate target-triggered L-RCA and the tailored γ-CD-P-MB was served as signal probe for L-RCA quantification. The developed SNP typing strategy was demonstrated by the SNP genotyping of β-thalassemia gene at position −28 (AAA mutated to AGA) in genomic DNA, which is one of the most commonly met mutations among the population in the southwest of China. The results revealed that both the wildtype and mutant targets were successfully discriminated. The present strategy is convenient with high sensitivity and selectivity, providing a good potential for practical applications.

2. Experimental

2.1. Reagents

All oligonucleotides were commercially synthesized by Takara Biotechnology Co., Ltd. (Dalian, China) and purified by high-performance liquid chromatography (HPLC). The sequences of these oligonucleotides were as follows:

Mutant target (T1): 5′-ATCAGGGCTGGGCATAGAAGTCAGGGCAGAGTA-3′ Wildtype target (T2): 5′-ATCAGGGCTGGGCATAAAAGTCAGGGCAGAGTA-3′ Padlock probe: 5′-PO_4-TATGCCCAGCCCTGTCTCACACTTCTTTCCACTCATTTCTCACACTTCTTTCCACTCATTTCTCACACTTCTTTCTGCCCTGACTTC-3′

RCA primer: 5′–AGAAATGAGTGGAAAG-3′

DNA fragment (P1) for construction γ-CD-P-MB: 5′-Pyrene-TCTCACACTTCTT- Pyrene-3′

Escherichia coli DNA ligase, *Phi*29 DNA polymerase and their reaction buffer were purchased from New England Biolabs (Beijing) Ltd. (MA, USA). dNTP was obtained from Takara biotechnology Co., Ltd. (Otsu, Japan). γ-CD stock solutions (100 mM) were prepared by dissolving the desired amount of the materials in doubly distilled water. All other reagents were of analytical reagent grade and were purchased from Sigma (Switzerland). All aqueous solutions were prepared using ultrapure water, which was obtained from the Nanopure Infinity™ ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, IA, USA) and had an electric resistance >18.2 MΩ. The SPSC buffer (1 M NaCl and 50 mM Na_2HPO_4, pH 7.5) was used for hybridization between P1 with L-RCA products.

2.2. Apparatus

All fluorescence measurements were carried out on an F-2500 fluorometer (Hitachi, Japan) equipped with an aqueous thermostat (Amersham) accurate to 0.1 °C. Excitation wavelength was set at 344 nm. Fluorescence emission spectra were collected using a bandwidth of 5 × 2.5 nm with a 700 V PMT voltage. All measurements were carried out at room temperature (25 °C) unless stated otherwise. Gels were scanned using a Tanon-2500R gel imaging system (Shanghai, China). All fluorescence emission images were recorded by a WD-9403 imaging system (Shanghai, China) with a transmitted ultraviolet light.

2.3. DNA hybridization kinetic and thermal profiles of γ–CD-P-MB

To study the kinetics and time-dependence of the interactions of P1 with γ-CD and subsequent the L-RCA product of target T1, the excimer fluorescence intensity of P1 at 475 nm was recorded. The pyrene excimer fluorescence of P1 (100 nM) was monitored for a few minutes. Then, an excess of γ-CD solution was added to the probe buffer and the excimer fluorescence was measured. After confirming that there was no change of fluorescence with time, L-RCA products of target T1 were added and the fluorescence intensity was recorded with time. For thermodynamic and temperature-dependent studies, two samples were prepared to determine the thermal profiles of P1 and γ-CD: one containing 2 μM P1 only, the other one containing 2 μM P1 and 10.0 mM γ-CD. All samples were performed in SPSC buffer. The temperature was controlled by refrigerating/heating circulators at every 5 °C from 10 °C to 95 °C.

2.4. L-RCA and γ-CD-P-MB based assay process

In a typical experiment, first, a 20 μL reaction mixture in buffer solution (30 mM pH 7.8 Tris–HCl, 26 μM NAD^+, 1 mM DTT, 4 mM $MgCl_2$), which contained 100 nM of padlock probe and a certain target oligonucleotide, was heated up to 95 °C and then cooled down to room temperature (For genomic DNA assay, the denatured mixture was immersed in ice-water immediately.). Then, bovine serum albumins (BSA, final concentration is 0.05%) and *E. coli* DNA ligase (final concentration is 0.5 U/μL) were added. The reaction mixture was incubated in a 37 °C water bath for 75 min. Second, the RCA reaction was carried out with the addition of 3 μL 10 × *Phi*29 DNA polymerase reaction buffer (500 mM pH 7.5 Tris–HCl, 100 mM $(NH_4)_2SO_4$, 40 mM DTT, 100 mM $MgCl_2$), 2 μL 1 μM primer solution, 8 μL 2.5 mM dNTPs, 0.5 μL 10 μg/μL BSA, 2 μL 10 U/μL *Phi*29 DNA polymerase. The polymerization reaction was carried out at 37 °C for 60 min. Subsequently, the resulting solution was incubated at 65 °C for 10 min to inactivate the *Phi*29 polymerase. After the resulting solution was cooled to ambient temperature, 10 μL γ-CD-P-MBs (2 μM P1, 10 mM γ-CD) were added and allowed to hybridize with the L-RCA products for 10 min prior to the fluorescence measurement.

2.5. Characterization of L-RCA product

15% Denatured polyacrylamide gel electrophoresis was used to directly characterize ligation product induced by padlock probe hybridizing with the target. Polyacrylamide gel was stained by SYBR Gold and run in 1 × TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.0) at room temperature. 16 μL of each sample was loaded into the lanes and performed at a constant potential of 80 V for 120 min. Gel electrophoresis was also used to prove the probe and polymerase-induced replication product. The electrophoresis experiment was performed by 0.5% agarose gels and run in 1 × TBE buffer at room temperature under a constant voltage of 100 V for 60 min with loading of 12 μL of each sample into the lanes. The electrophoresis experiment was carried out with Western Electrophoresis apparatus (BIO-RAD, USA).

3. Results and discussion

3.1. SNP genotyping principle

As illustrated in Fig. 1, the SNP genotyping approach was based on stemless molecular beacon and ligation-rolling circle amplification (L-RCA). Firstly, the stemless DNA molecular beacon γ-CD-P-MB was constructed as a signal probe. Different from classic MB probes, which need a 15 – 25 bases loop together with a 5–7 mer stem to provide an appropriate balance for the formation of a hairpin structure [36], γ-CD-P-MB with 13 bases long was formed by bounding the end-labeled pyrene molecules of short oligonucleotides in the γ-CD cavity. This stemless molecular beacon avoids conformational constrained Watson-Crick base paired-stem and greatly shortens the length of the oligonucleotides, resulting in increased flexibility in probe design.

For this SNP genotyping method, an 87 bases padlock DNA probe was used as recognition probe. The fifteen 3′-terminal and fourteen 5′-terminal bases of the padlock probe was designed to be perfectly complementary to the sequences of mutant target (T1). The padlock probe was firstly hybridized with target DNA. Then, the 3′–OH and 5′–PO_4 ends of the padlock probes were covalently joined to form a circular DNA template by *E. coli* DNA ligase only when the base at the 3′-end of the padlock probe was perfectly matched to the target. RCA was subsequently initiated by the high-displacement activity of the *Phi*29 DNA polymerase in the presence of a short primer and dNTPs. The long ssDNA product containing repetitive target sequence units was generated in large quantities and served as an excellent template for binding of γ-CD-P-MB. As a result, the two pyrene molecules embedded in the γ-CD cavity were moved away from each other, causing a decrease in the pyrene excimer fluorescence at 475 nm. Thus, through the fluorescence change of pyrene excimer, SNP might be successfully identified.

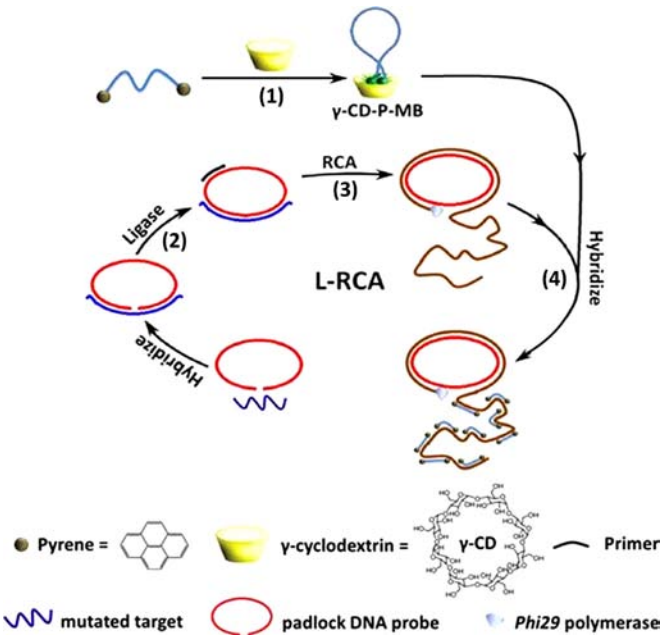

Fig. 1. Schematic illustration of SNP genotyping strategy based on L-RCA and γ-CD-P-MB. (1) The formation of γ-CD-P-MB; (2) hybridization between padlock probe and target and ligation; (3) RCA reaction and (4) hybridization between L-RCA product and γ-CD-P-MB.

3.2. Characterization of γ-CD-P-MB formation

Following the design, the γ-CD-P-MB construction was firstly investigated. Fig. 2A shows the fluorescence emission spectra of P1 solution in the absence and the presence of γ-CD at room temperature. In the absence of γ-CD, P1 exhibited low-intensity structureless band with an emission maximum center at 475 nm, typicality of pyrene excimer emission. The formation of excimers of aromatic hydrocarbons is restricted to a parallel, cofacial configuration with an interplanar distance of 3–4 Å [33], so the pyrene molecules labeled on both ends of P1 keep random state and shows low probability for the formation of pyrene excimers. After the addition of the γ-CD, the fluorescence intensity at 475 nm was enhanced hundreds times. This fluorescence emission change of P1 induced by γ-CD suggested that the two pyrene molecules of P1 encapsulates in the γ-CD cavity (internal diameter = 8.5 Å) to form pyrene excimers. The reason for this is that the γ-CD can modulate the space proximity of the end-labeled pyrenes through cyclodextrin/pyrene inclusion interaction. Subsequently, the stability of γ-CD-P-MB probe was further determined by melting temperature (*Tm*) measurement. The correlation between the Tm value and the fluorescence emission of γ-CD-P-MB was examined in the temperature range from 10 to 95 °C. As the temperature of a solution containing γ-CD-P-MB was slowly raised, the excimer emission decreased (Fig. 2B). The Tm value, defined as the temperature at which the excimer fluorescence of γ-CD-P-MB probe reaches 50% of its maximum value, was estimated to be 56 °C. High value for Tm indicated the thermal stability of γ-CD-P-MB. All these results clearly demonstrated that the γ-CD-P-MB, as a fluorescence signal probe, was well constructed.

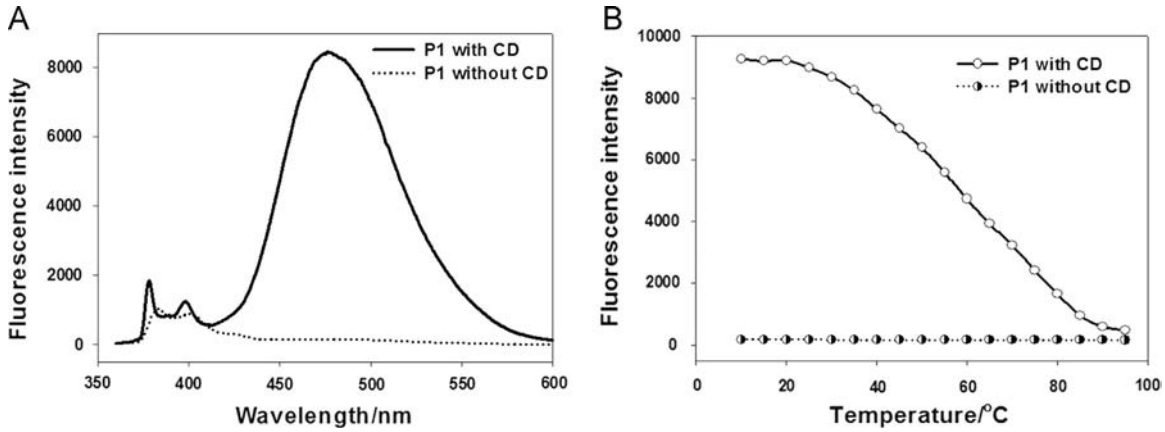

Fig. 2. (A) Fluorescence emission spectra of aqueous P1 solution in the absence and the presence of γ-CD; (B) excimer fluorescence intensity changes of P1 (2 μM, λex=344 nm) as a function of temperature in the absence and the presence of 10 mM γ-CD.

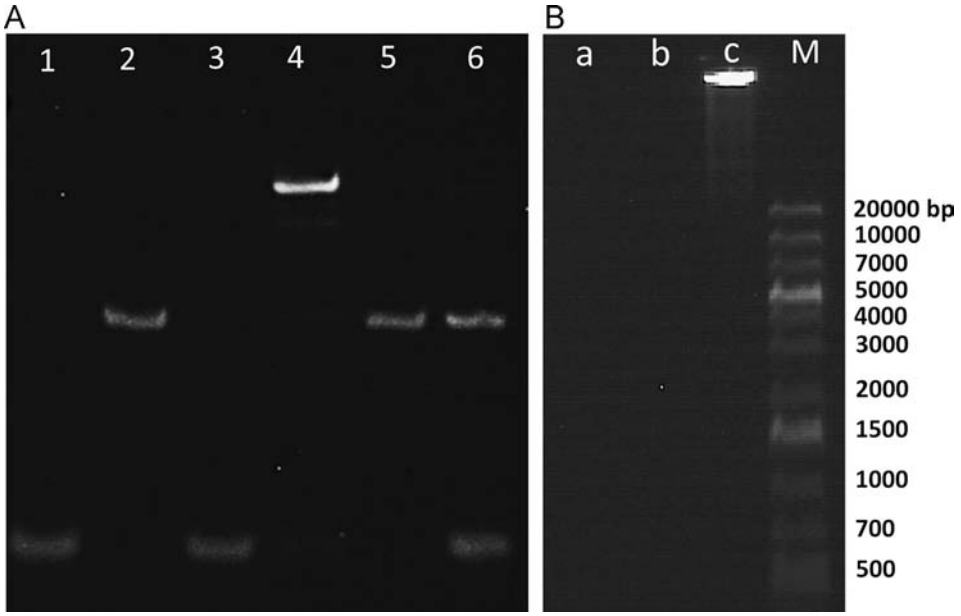

Fig. 3. Electrophoretic analysis of oligonucleotides samples. (A) Denaturing polyacrylamide gel images of ligation products. Lane 1: T1, Lane 2: padlock probe, Lane 3: T2, Lane 4: ligation products of T1, Lane 5: ligation products without T1 or T2, Lane 6: ligation products of T2. (B) Agarose gel (0.5%) electrophoresis images obtained in L-RCA reaction. Lane a: L-RCA products of T2, Lane b: L-RCA products without T1 or T2, Lane c: L-RCA products of T1 (experimental group), Lane M: a marker of 500–20000 bp.

3.3. Characterization of L-RCA reaction

Moreover, in this strategy for SNP genotyping, the specialty and sensitivity was offered by L-RCA. In our work, mutant target DNA (T1) was designed to match with the padlock probe perfectly, allowing circularization of the padlock probe in the presence of DNA ligase. The circularization product could then serve as template for RCA reaction. The long ssDNA product with repeated sequences could be further hybridized with γ-CD-P-MB, leading to read out signal amplified. Thus, the L-RCA reaction was characterized with gel electrophoresis. Generally, *E. coli* DNA ligase is fairly critical for the base match at the nick, and its fidelity is better than the other DNA ligase studied before, which is more tolerant to base pair mismatch on both termini of the nick for successful ligation [37,38]. The high fidelity of *E. coli* ligase on recognition of base pair mismatch was verified through denatured polyacrylamide gel electrophoresis. Fig. 3A shows an image of the gel recorded, lane 1, lane 2 and lane 3, was T1, padlock probe and T2. Lane 4 was the mixture of padlock probe, T1 and *E. coli* DNA ligase after incubating 75 min. lane 5 was the mixture of padlock probe and E. coli DNA ligase. Lane 6 was the mixture of padlock probe, T2 and E. coli DNA ligase after incubating 75 min. In lane 4, we could easily find that one obvious band, which migrates at the backmost position in all of bands. It was the slow-migrating circularized oligonucleotide. However, two obvious bands appear at the position corresponding to target band, which is the T2, and the other migrates at the position similar to the band in lane 2, which is the padlock probe. The results showed that the ligation reaction was happened and strictly dependent on correct sequence complementarity of the padlock probe. The subsequent RCA reaction was also characterized by gel electrophoresis in a 0.5% agarose. As shown in Fig. 3B, the expected high molecular weight of RCA product (exceed 20,000 bp) was confirmed in lane c in the presence of T1. However, if T1 is absent or T2 is present, no bands could be observed due to the lack of circularized oligonucleotide as template of RCA. This result revealed that the nick between the two adjacent probes was ligated by *E. coli* DNA ligase, and the long DNA was produced by RCA in the presence of T1.

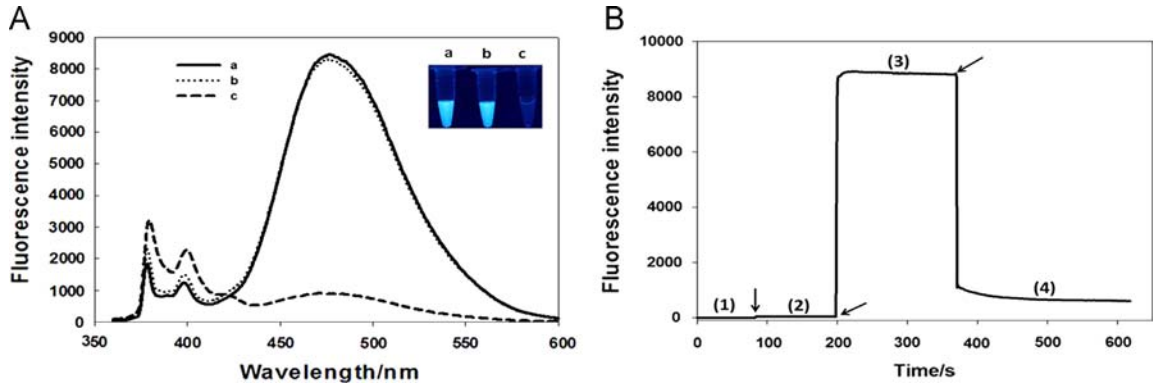

Fig. 4. (A) Fluorescence emission spectra (λex = 344 nm) of the γ-CD-P-MB after incubation with the reaction mixture without target (a) and with T2 (b) or T1 (c). The concentration of the padlock is 50 nM, and the concentrations of T1 and T2 are 1 nM, respectively. The inset showed the corresponding solution color under ultraviolet radiation. (B) Real-time fluorescence records of P1 (2 μM) at 475 nm upon addition of γ-CD (10 mM) and subsequent L-RCA products with T1. For the measurement, four steps were distinguished: (1) the cuvette was filled with SPSC buffer solution, (2) P1 was introduced in the cuvette, (3) γ-CD was added, and (4) L-RCA products of T1 were added. The transition between each regime is marked with an arrow.

3.4. Feasibility test of the γ-CD-P-MB and L-RCA based assay for SNP genotyping

After characterizing the γ-CD-P-MB formation and L-RCA reaction, the feasibility of L-RCA combined with γ-CD-P-MB for SNP genotyping was then demonstrated by measuring and imaging the fluorescence change of γ-CD-P-MB. The fluorescence emission spectra of γ-CD-P-MB solution under exaction of 344 nm were recorded. The concentrations of the padlock probe, *E. coli* DNA ligase and *Phi*29 polymerase was 50 nM, 0.5 U/μL and 0.6 U/μL, respectively. T1 and T2 were both 1 nM. In the following three experiment groups, padlock probe, *E. coli* DNA ligase and *Phi*29 polymeras were all present in the L-RCA reaction mixture. The fluorescence characteristics and corresponding fluorescence images of the resulted solutions under UV irradiation are shown in Fig. 4A. When no target DNA was present, the L-RCA reaction mixture incubated γ-CD-P-MB exhibited strong fluorescence at 475 nm (curve a), relating to pyrene excimer emission due to the hairpin-structure of γ-CD-P-MB. The fluorescence image presented intense blue-white signal under ultraviolet radiation. After T2 was added, almost no change has been observed for the fluorescence emission spectrum of L-RCA reaction mixture incubated γ-CD-P-MB (curve b). However, the excimer fluorescence intensity of γ-CD-P-MB greatly decreased when T1 was present. It is because that the two pyrenes labeled on the DNA were away from γ-CD cavity and separated as a result of the hybridization of γ-CD-P-MB with the repetitive sequence units of L-RCA products. Simultaneously, from the fluorescence image, it was observed that only very weak blue fluorescence signal was observed in the presence of T1. The process of the SNP genotyping based on L-RCA combined with γ-CD-P-MB was also characterized by real-time fluorescence recording. Real-time fluorescence of P1 (2 μM) at 475 nm upon addition of γ-CD (10 mM) and subsequent L-RCA products in the presence of T1 (1 nM) were measured. As depicted in Fig. 4B, the γ-CD-P-MB was formed fast at room temperature and achieved equilibrium with the maximum excimer intensity. When L-RCA products of T1 were added to the solution of γ-CD-P-MB, the excimer fluorescence drastically decreased within few minutes, indicating the formation of duplex and separation of the two pyrene molecules. These results confirmed that the γ-CD-P-MB and L-RCA based assay for SNP genotyping could be carried out.

3.5. Optimization of the SNP assay conditions

In the following, we devoted to assessing possible variables that could influence the result of SNP typing. The ligase reaction time and the RCA duration were investigated. To estimate the effect of ligase reaction time on the efficiency in generating circular probes, γ-CD-P-MBs were added after RCA reaction. Then, the effect of ligase reaction time could be estimated from the fluorescence change of the reaction mixture at 475 nm. As observed from Fig. S1**A**, the fluorescence intensity at 475 nm was no longer decreased after the ligation time lasted for 75 min, indicating that the reaction has reached equilibrium. So, 75 min was chosen as the ligase reaction time for circular probe formation in our experiment.

For RCA reaction, in theory, more complementary copies of the circular template are generated with the elongation of RCA reaction time; stronger signal amplification will be produced. So the effect of RCA reaction time on the fluorescence signal was examined. The result was shown in Fig. S1B. Under a higher concentration of *Phi*29 polymerase and substrates, the optimal RCA duration was found to be at about 1 h from the plot of the excimer fluorescence change at 475 nm versus RCA duration. RCA duration longer than 1 h actually did not yield significant decrease for the product signal. This might be due to the fact that the RCA reaction had reached equilibrium and the activity of *Phi*29 polymerase was substantially inhibited by the RCA products after 1 h. So 1 h was chosen as the RCA and enzyme digestion time.

3.6. SNP quantitative analysis

Having established the methodology, we applied it to detect and quantify the DNA targets under the optimal conditions. Fig. 5A depicts fluorescence emission spectra of the assay upon the addition of T1 at different concentrations. The plot of fluorescence intensity versus T1 concentration is shown in Fig. 5B.The fluorescence intensities decreased remarkably when the concentration of T1 was raised from 0 fM to 1 nM, and there was a fairly good linear relationship between the fluorescence intensity and the concentration of T1 in the range from 50 fM to 500 fM. The regression equation was expressed as $F = 8617.3 - 0.837C_{T1}$ (C_{T1} represents the concentration of T1, fM; correlation coefficient $R = 0.9974$) and the detection limit, which was calculated in terms of three times the signal-to-noise level, is ca. 40 fM.

3.7. SNP typing in constructed DNA samples

Specificity was one of the most significant factors for SNP genotyping, because it reflected the ability of the assay to avoid false negative or false positive readouts. To verify the single-base specificity of our assay, the target oligonucleotides of T1 and T2 were mixed at various ratios with a total concentration of 1 nM.

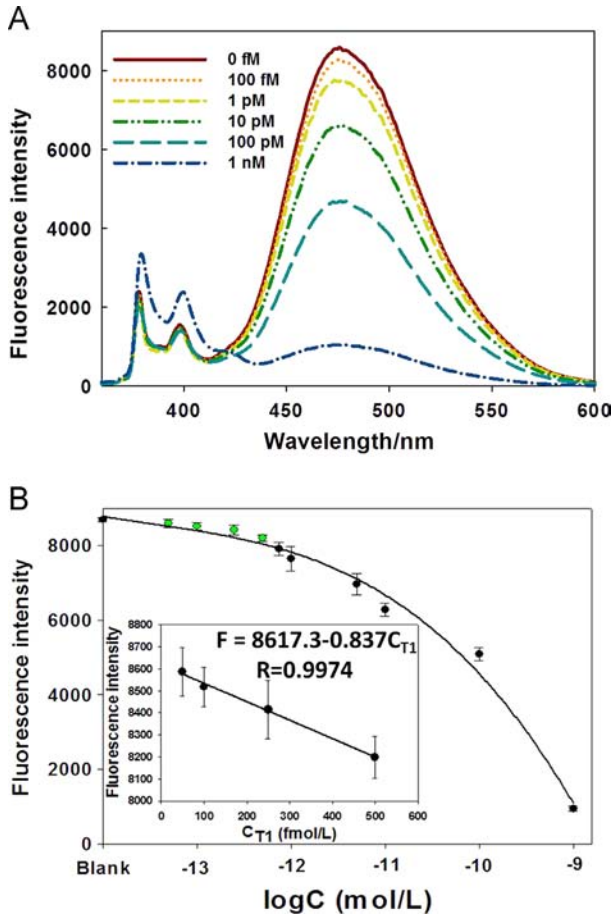

Fig. 5. Quantitative analysis of the target DNA T1 using fluorescence spectrophotometry. (A) Fluorescence emission spectra recorded at different concentrations of T1. (B) Fluorescence intensity changes upon the addition of different concentrations of T1. The inset of (B) displays the linear relationship between fluorescence intensity and the T1 concentration. The error bars are standard deviations of four repetitive measurements.

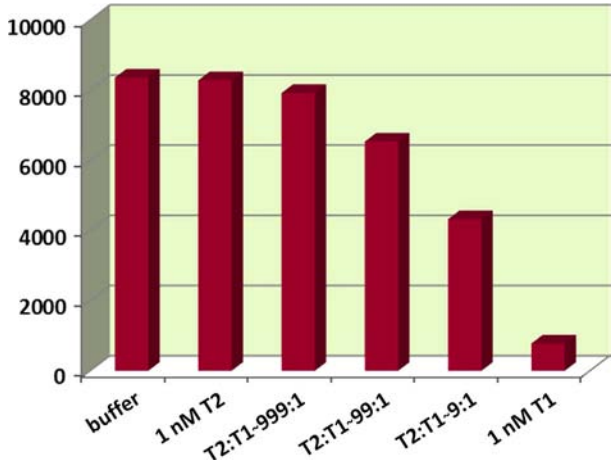

Fig. 6. Effect of the mutant DNA (T1) to wildtype DNA (T2) ration on the detection of SNP. The total concentration of mutant DNA and wildtype DNA was 1 nM.

From the results of Fig. 6, the change of fluorescence intensity decreased with the increase of T1 percentage in the sample solution. Obviously, when the mutant target was present, even if the ratio of the wildtype target to the mutant was 999:1 (1 pM T1 in the mixture), decrease was still significant and higher than that for the control experiment using 1 nM T2. Furthermore, we observed that fluorescence decrement was correlated to the concentration of T1. The result indicated that the present approach had high specificity for detecting a trace mutation among a large quantity of wildtype targets, which granted the assay great potential in clinical applications.

4. Conclusions

In summary, combined with ligation-rolling circle amplification and a novel stemless molecular beacon γ-CD-P-MB, a highly selective, sensitive and rapid technique was successfully used for SNP genotyping. The use of *E. coli* DNA ligase furnished this technique with desirable specificity in identification of SNP, and the utilization of RCA allowed highly efficient amplification to create a great abundance of repeated sequences for hybridization with stemless γ-CD-P-MB signal reporter. With such excellent amplification signal and extreme fidelity, the present method could achieve a detection limit as low as 40 fM and positive discrimination for low-abundance −28 β-thalassemia gene in the presence of 1000 times more wildtype targets. In addition, it is worthwhile to point out that no sophisticated instrument is needed, and it took less than 3 h to finish an assay. These advantages endow the SNP identification strategy with a great potential in the pathogenic diagnosis and association studies.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.014.

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