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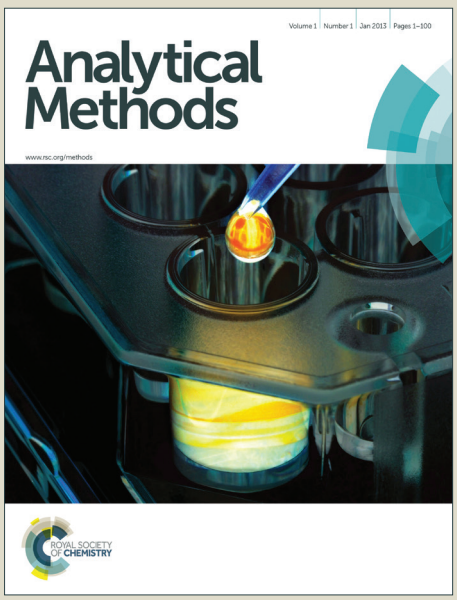

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Melting temperature of molecular beacon as an indicator of ligase detection reaction for multiplex detection of point mutations†

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The multiplex detection of point mutations plays an increasingly important role in clinical diagnosis of genetic-based diseases. The present study reports a novel technology for the analysis of multiple point mutations based on ligase detection reaction (LDR) and melting curve assay. In this method, LDR was utilized to identify mutations using the high selectivity of Taq DNA ligase. While the probes matched the target sequence perfectly, the allele-specific discriminating probe (5' labeled with fluorophor) and common probe (3' modified by quencher) will joined with each other. Through the special design, the ligation product can then form a molecular beacon. Finally, melting temperature (Tm) of the molecular beacon was measured by melting curve assay. The mutation type was identified through the melting curve and Tm value. For multiplex detection, the molecular beacons with different Tm were achieved by changing the length or GC content of the arm sequences and then presented to the different mutation sites. Three common point mutations of the α-globin gene in thalassemia disease were identified simultaneously using this approach. The Tm achieved by the assay for each of the three mutation sites [Cd122 (C>G), Cd125 (T>C) and Cd142 (T>C)] were 73℃, 71℃ and 60℃, respectively. The veracity and reproducibility of the method was evaluated by clinical samples which were validated by sequencing. In summary, this method, based on LDR and melting curve assay, provides a promising tool for detection of gene mutations.

Introduction

Mutation detection plays an increasingly important role in the fields of genetic-based disease diagnosis[1, 2]. Detection of genetic mutations has been widely applied in disease prevention, molecular diagnosis and clinical genetic testing[3, 4]. Mutation detection in the Chinese population, for example, has shown that more than 50 mutations can cause thalassemia, which is one of the most common monogenic diseases in the world[5, 6]. Therefore, development of new technologies that could detect multiple mutations simultaneously plays a crucial role in genetic-based disease diagnosis.

To date, a variety of approaches, based on allele discrimination strategies, have been applied to identify and visualize point mutations. Conventionally, an assay is usually performed by gel electrophoresis and autoradiography, both of which are time-consuming and technically challenging[7]. In recent years, many other technologies have been developed for allele-specific discrimination, such as allele-specific probe hybridization[8], allele-specific primer extension via PCR[9], enzymatic cleavage[10, 11] and oligonucleotide ligation[12]. Among these assays, the ligation detection reaction (LDR) is popular for use in the discrimination of point mutations because of its high specificity, low cost , and ease of operation[13, 14].

Developed by Francis Barany[15], LDR relies on the high selectivity of the thermostable ligase enzyme and offers a simple and feasible alternative platform for mutation detection. For multiplex detection, the LDR has significant advantages in that it allows sets of probes for a number of mutation sites to be multiplexed together, enabling several mutations to be detected simultaneously[16]. However, its application has been limited because LDR products are mostly detected by gel electrophoresis separation or heterogeneous analysis, such as

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denaturing gradient gel-electrophoresis[17], DNA microarray[18]、capillary electrophoresis[19]. Some of these are time consuming, complicated to operate and costly.

Here, we report a novel strategy for the detection of multiple mutations based on LDR and melting curve assay, using molecular beacons as sensors for output signals of LDR products and using three common point mutations of the α-globin gene in thalassemia disease as a model[5]. We hereby demonstrate that this new method, based on LDR and melting curve assay, was able to accurately detect the three mutations simultaneously; furthermore, using 4 or 5 fluorescence channels and more molecular beacons with different melting temperatures, more than 20 other mutations were also identified.

Material and methods

Oligonucleotides and reagents

The oligonucleotide sequences of all the LDR probes, templates and template complements used in these experiments are shown in Table 1. All oligonucleotides were synthesized and purified by Takara Biotechnology Co., Ltd (Dalian, China). For identifying each point mutation, two allele-specific ligation probes were designed, a discriminating probe and a common probe. The discriminating probe was fluorescently labeled with HEX (fluorophore) at its 5'-end of a stem sequence and a discriminating base at its 3'-end. The common probe was designed to have a stem sequence that was end labeled with BHQ1 (quencher) at its 3'-end and phosphorylated at its 5'-end. The stem sequences of the probes were complementary to each other. Melting temperatures (Tm) and structures of all ligation products were designed and evaluated with the help of DNA probe design software (Zucker folding program, http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form)[20]. For designing ligation probes, we make sure that the secondary structures with highest thermodynamic stability were formed by the hybridization of stem sequences. The molecular beacon structures were predicted using Zucker folding program by adjusting the folding temperatures to form only one secondary structure(secondary structure with highest thermodynamic stability). And then, the Tms of molecular beacons formed by ligation products were calculated. Taq DNA ligase was purchased from New England Biolabs (Ipswich, MA, USA). LA Taq DNA polymerase, GC buffer Iand dNTP mixture were purchased from Takara Biotechnology Co., Ltd (Dalian, China). gDNA samples were obtained from the Department of Medical Genetics at Southern Medical University (Guangzhou, China). The research ethics committee of Southern Medical University approved the study protocol. DNA was extracted from peripheral blood by standard phenol/chloroform methods. The genotypes of each samples were identified by DNA sequencing.

PCR amplification of genomic DNA

PCR amplification was performed in a 50 μL system with 1×GC I buffer [20 mM Tris-HCl (pH 8.8) with 10 mM $(NH_4)SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100 and 10 mM KCl], 250 μM deoxyribonucleotide triphosphate (dNTPs), 400 nM forward and reverse primers (200 nmol for each primer), 2.5 Units LA Taq DNA polymerase, as well as ~ 20 ng of genomic DNA extracted from the samples. The sequence of the forward

Table 1 Oligonucleotides used in this assay[a]

Name	Sequence(5'-3')
Wild-type template CD122CD125 (122-125W)*	CCCGCCGAGTTCACCCCTGCGGTGCACGCCTCCCTGGAC AAGTTCCTGGCTTCTGTGAGCACCGTG
Mutant template CD122(122M)	CCCGCCGAGTTCACCCCTGCGGTGCAGGCCTCCCTGGA CAAGTTCCTGGCTTCTGTGAGCACCGTG
Probe CD122(122H)	HEX-ACTTGTCCAGGGAGGCC
Probe CD122(122Q)	p-TGCACCGCAGGGGTGGACAAGT-BHQ_1
Mutant template CD125(125M)	CCCGCCGAGTTCACCCCTGCGGTGCACGCCTCCCCGGA CAAGTTCCTGGCTTCTGTGAGCACCGTG
Probe CD125(125H)	HEX-GCGTGCAAAAAAGCCAGGAACTTGTCCG
Probe CD125(125Q)	p-GGGAGGCGTGCACGC-BHQ_1
Wild-type template CD142(142W)	GTGAGCACCGTGCTGACCTCCAAATACCGTTAAGCTGG AGCCTCGGTAGCCGTTCCTCC
Mutant template CD142(142M)	GTGAGCACCGTGCTGACCTCCAAATACCGTCAAGCTGG AGCCTCGGTAGCCGTTCCTCC
Probe CD142(142H)	HEX- ACTGAAACCGAGGCTCCAGCTTG
Probe CD142(142Q)	p-ACGGTATTTGGAGGTCAGCTTTCAGT-BHQ_1

[a] Templates and probes used in this paper were designed according to ref[20]. with some modifications. These designed sequences were used to detect Cd122 (C>G), Cd125 (T>C), Cd142 (T>C) point mutations of α-globin gene associated with thalassemia disease. The circled base in 122M, 125M and 142M indicates the mutant bases. p in 122Q, 125Q and 142Q represent 5'-phosphorylation. HEX is Hexachioro fluorescrin. BHQ1 is Black Hole Quencher-1.The bases

underlined indicates the arm sequence. * the mutation sites of Cd122 (C>G) and Cd125 (T>C) are closed, so used the common wildtype template.

primer was 5'-TGG AGG GTG GAG ACG TCC TG-3' and the sequence of the reverse primer was 5' -CCA TTG TTG GCA CAT TCC GG-3'. Amplification was achieved by the following thermocycler program: (1) 95℃ for 5 min (2) 95℃ for 45 s (3) 60℃ for 30s (4) 72℃ for 1 min 20s (5) repeat steps 2-4 35 times (6) final extension at 72℃ for 5 min. The resulting products were confirmed by agarose gel assay and directly used for subsequent LDR reaction without purification.

LDR reaction and melting curve assay

For multiplex LDR reaction, the 20 μL reaction mixture contained 5 nM of synthetic oligonucleotide targets or PCR products, 50 nM of each LDR probes, Taq ligation buffer [20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD+, 0.1% Triton X-100] and 4 U of Taq DNA ligase. The LDR reactions used the following thermocycler program: (1) 95℃ for 3 min (2) 95℃ for 30 s (3) 50℃ for 5 min (4) repeat steps 2-3 20 times. The ligation solution was then heated at 98℃ for 20 min to inactivate DNA ligase and then cooled to 45℃. Melting curve assays were carried out on the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with a heating rate of 0.2 ℃/s starting at 45 ℃. Fluorescence values of each tube were measured with excitation at 535 nm and emission at 556 nm.

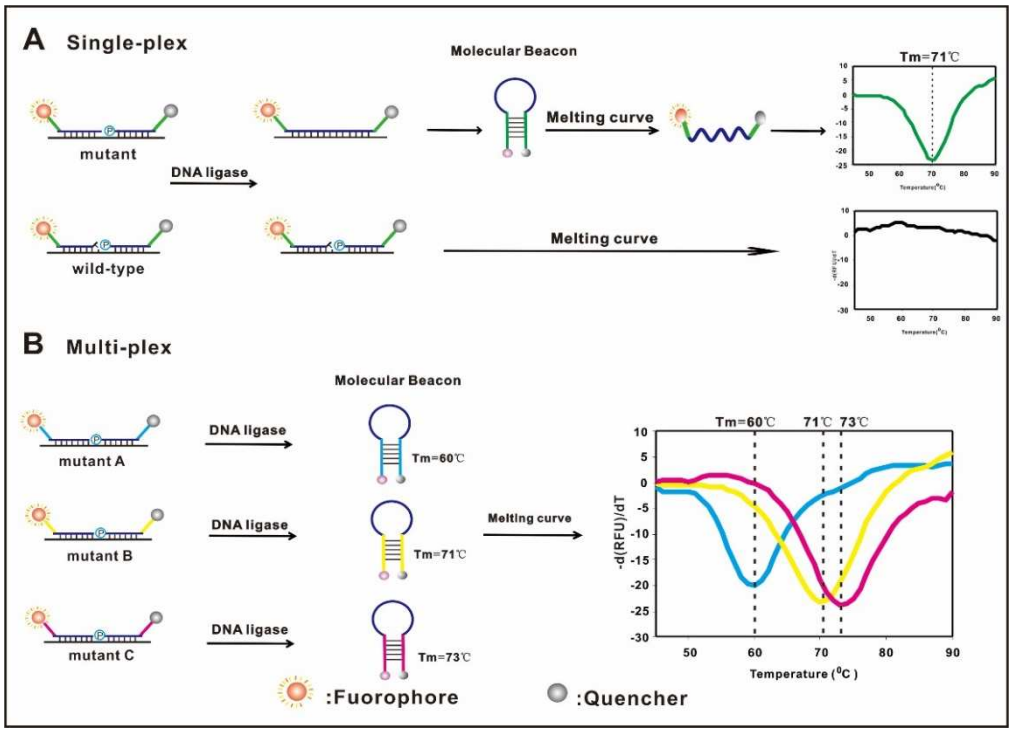
Gel shift assay

Denaturing polyacrylamide gel electrophoresis was used to confirm the multiplex LDR reaction. In order to perform denaturing polyacrylamide gel electrophoresis, ligation mixtures similar to that mentioned above, but containing 50 nM of synthetic oligonucleotide targets, 500 nM of each LDR probe and 20 U of Taq DNA ligase, were prepared. After the reaction, 5 μL of the reaction products were mixed with 5 μL of loading buffer [62.5% formamide, 0.4 M formalhedyde, 1.25 × MOPS Buffer, 0.02% Xylene Cyanol FF, 0.02% Bromophenol Blue], denatured at 90℃ for 10 min and chilled rapidly on ice prior to electrophoresing on an 8M urea-16% polyacrylamide gel. In this study, the discriminating probes were labeled with fluorophores, enabling detection of the ligation products without the gel being stained.

Results

Analytical principle

The implementation of simultaneous genotyping of multiple DNA targets requires a biocode that could uniquely discriminate the identity of DNA targets and the presence of point mutations. In our methodology, we exploited melting temperatures as natural biocodes for DNA targets, based on pairs of tailor-made oligonucleotide probes flanking the mutation site that could then form molecular beacons with different melting temperatures while they were covalently joined to each other. The assay protocol is outlined in Scheme 1A. The discriminating probe labeled with fluorophore and the common probe modified with quencher hybridized with the target DNA at an adjoining position. Since the probes we designed were only perfectly complementary to the mutant template



Scheme 1 Illustration of multiplex mutation detection utilizing ligase detection reaction coupled with melting assay of molecular beacons which formed by LDR products..

DNA, Taq DNA ligase was able to specially catalyze the formation of a phosphodiester bond between the juxtaposed 5'-phosphate and 3'-hydroxyl groups of the above probes to form a single oligonucleotide in the presence of mutant target DNA, but no ligation occurred for probes associated with the wild-type template. Using thermal cycling of thermostable DNA ligase, the allele discrimination reaction proceeded repeatedly, thus providing a route for the amplification of the ligation products in the perfectly complementary system. Because the 5' stem sequence of the discriminating probe and the 3' stem sequence of the common probe were complementary to each other, the ligation products were able to form the structures of molecular beacons. Free molecular beacons in solution can have at least two distinct conformations: stem-loop (low temperature) and random-coil (high temperature). Fluorescence resonance energy transfer (FRET), the fluorescence intensity of the fluorophore, was weak in the stem-loop conformation but strong in the random coil random coil conformation. Finally, a melting curve assay was executed for detecting the Tm of the molecular beacons folded by ligation products. In the process of the melting curve assay, the fluorescence intensity was gradually increased in line, along temperature increments so that the conformation of molecular beacon was changed from stem-loop to random-coil. Through software calculations, we identified the ligation products with the determined Tm, therefore determining the melting curve and Tm of the molecular beacon. .

For the multiplex assay, the molecular beacons which had been designed to have different Tms were used to represent the different mutation sites, as shown in Figure 1B. The molecular beacons with different Tms was achieved by changing the lengths and GC contents of the stem sequences.

Analysis of mutation in synthetic oligonucleotide targets

In order to evaluate the feasibility of the method, we firstly analyzed the mutations in synthetic oligonucleotide templates (Table 1) to avoid the effects of variability between real DNA samples on the evaluation results. Wild-type templates (122-125W, 142W) and mutant templates (122M, 125M and 142M) were derived from variants of the human HBA2 gene sequence. Fig.1 displays the results obtained from wild-type template and mutant template of three mutation sties: (A) Cd122 (C>G), (B) Cd125 (T>C), (C) Cd142 (T>C). It was observed that the inverted peaks of melting curves only appeared in mutant templates which were perfectly matched to the ligation probes. The Tms, as the biocode of the three mutation sites, were: 73 ℃， 71 ℃ and 60 ℃， respectively. The values of –d(RFU)/dT, corresponding to the peak temperatures obtained from the analysis of the three perfectly matched mutant targets (-18.49, -20.96 and -20.06, respectively), were significantly higher than those of wild-type targets with a single-base mismatch. The data obtain for a multiplex reaction is shown in Fig.1D. Similar to the results of a single-plex reaction, the inverted peaks of the melting curve were only presented in the reaction with mutant templates. The melting temperatures (73 ℃, 71 ℃ and 60 ℃) of the three mutation sites were identified by the assay with pairs of LDR probes. Since the Cd 122

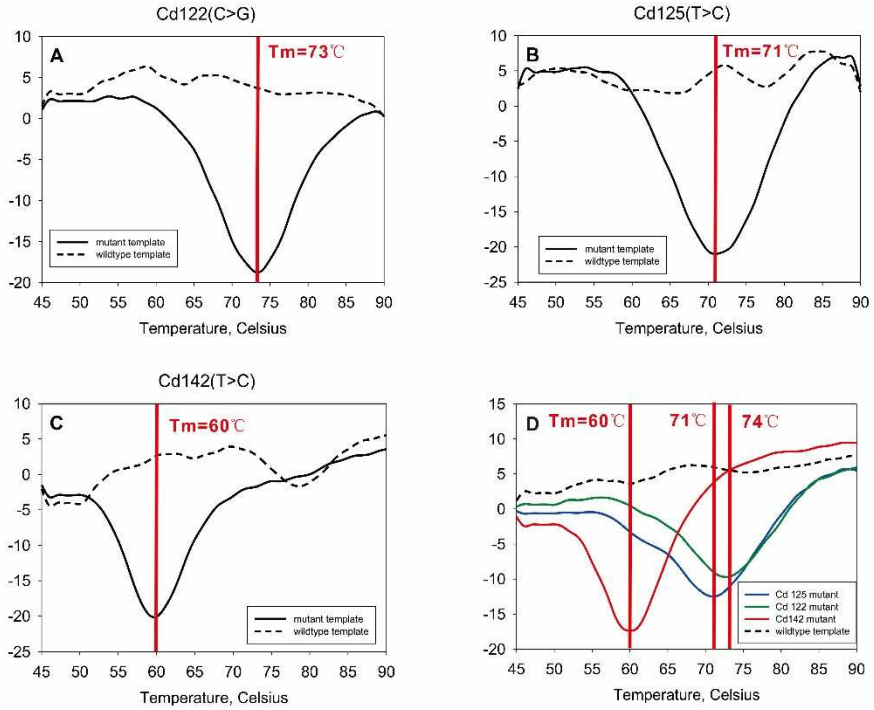


Fig.1 The melting curve assay results of the single-plex or multiplex LDR. (A-C): Results of single-plex LDR for each mutation site using wild type or mutant synthetic oligonucleotides as a template. (A) Cd 122 (C>G), (B) Cd 125 (T>C) and (C) Cd 142 (T>C). The concentrations of each of the three pairs of LDR probes are 50 nM, and 5 nM templates contained wild type and mutant template. (D): The melting curve assay result

using all of the three pairs of LDR probes. The concentrations of each of the three pairs of LDR probes are 50 nM, and 5 nM templates containing wild type and mutant template were used for the reaction.

site was close to the Cd125 site, and the hybridization sequences of the LDR probes were partially overlapping, the -d(RFU)/dT values of templates M1 and M2 [with mutation Cd122 (C>G) and Cd125 (T>C)] (-9.591 and -12.54) were significantly reduced compared with the values of the single-plex reaction where only one pair of LDR probes was hybridized to a template. The results of the multiplex assay revealed that the complicated conditions have no effect on the specificity of the Taq DNA ligase reaction and melting character of the ligation products.

Gel image

To further validate the specificity of Taq DNA ligase in the multiplex reaction, a gel electrophoresis assay was carried out to confirm evidence of the ligation process. Since the gel was prepared without any staining, only the probe labeled with fluorophore could be detected. As shown in Fig.2, three bright bands, which presented in all lanes, represented the discriminating probes of each mutation. Lane 1 (template-free ligation product control) and lane 2 (wild-type template ligation product) were not present in the product band, since the Taq DNA ligase was unable to work in the absence of template or a discriminating probe that matched the template. Moreover, obvious product bands appeared in ligation products with mutant templates (lane 3, lane 4 and lane 5). At the same time, the band of each of the discriminating probes was darker than the band in line 1 and line 2. Thereby, the results of gel electrophoresis were consistent with the results of the melting curve assay.

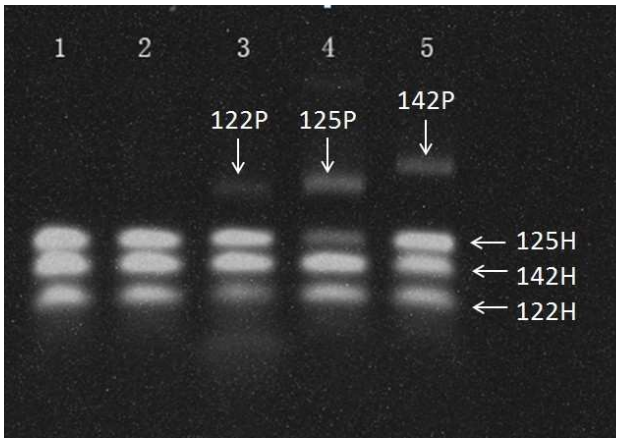


Fig.2 Multiplex ligation products were separated by 16% polyacrylamide gel electrophoresis with 8M urea, and DNA was screened without any staining.(Lane 1) Template-free ligation products control, (Lane 2) Wild-type template ligation products, (Lane 3-5) Ligation products of template with 122M, 125M and 142M, respectively. 122H, 125H and 142H are the discriminating probes labeled with HEX for detected mutant Cd122, Cd125 and Cd142, respectively. 122P, 125P and 142P are the ligation products formed by joining the discriminating probes and common probes which were used to detect the mutant Cd122, Cd125 and Cd142, respectively.

Analysis of genomic DNA

In order to further validate that the LDR/melting curve assay could be applicable to clinical samples, several human genomic DNA samples (5 samples per genotype) were collected with genotypes characterized by DNA sequencing (see ESI†). PCR, multiplex LDR and melting curve assay were all performed as described in the Materials and Methods section. This was followed by PCR on these samples to obtain 1314 bp amplicons containing the full sequence of the HBA_2 gene. The products of PCR were then used directly in the multiplex LDR/melting curve assay. The results obtained from these samples, using the presented method, are shown in Fig.3. The results show that no inverted peak of melting curve appeared (Fig.3A), indicating that the sample is wild-type. Inverted peaks of melting curve are shown in Fig.3B-C indicating that all these samples are mutants. The Tms obtained from the three mutants [Cd122 (C>G), Cd125 (T>C) and Cd142 (T>C)] were 73.40±0.548℃, 70.80±0.447 ℃ and 59.80±0.447 ℃ , respectively. The coefficients of variation (CV,) of the Tms obtained from the three mutants [Cd122 (C>G), Cd125 (T>C) and Cd142 (T>C)] were 0.75, 0.63 and 0.75%, respectively.

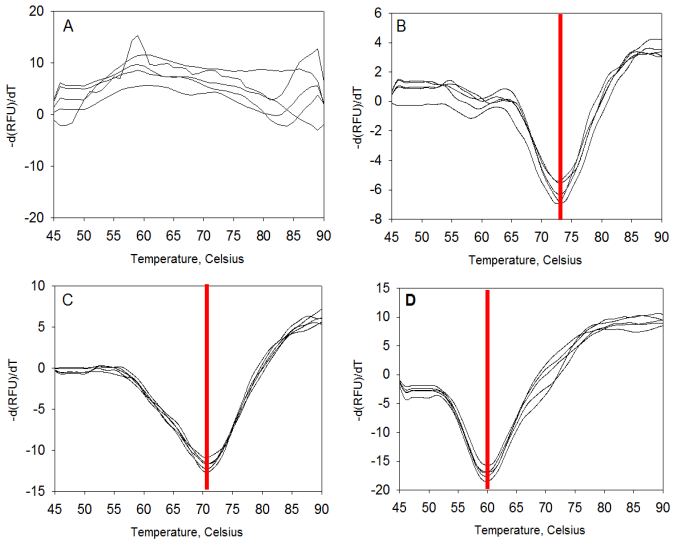


Fig.3 The results of multiplex LDR and melting assay of a genomic DNA sample. Five samples of each genotype (A) Wild type, (B) Cd 122 (C>G), (C) Cd 125 (T>C) and (D) Cd 142 (T>C).

Discussions

The present study reports a novel approach for the detection of multiple mutations based on LDR and melting curve assay. This system allows accurate discrimination of multiple mutation sites and does not require a microarray format or capillary electrophoresis. Different from microarray and capillary electrophoresis, the new strategy utilizes a melting curve assay as a signal acquisition platform, and exploits the Tm values of molecular beacons, which are formed by ligation products replacing the length or the label of ligation products, as signals. The melting curve assay is rapid and convenient, using a closed single-well system, without the requirement of any post-LDR handling of solutions. Afterwards, the mutation

type could be identified through the Tm value calculated by the software, thereby significantly reducing the time-consuming work of hybridization, elution and electrophoresis which are the requirements of microarray and capillary electrophoresis[21, 22].

In the establishing of our method, three mutations were simultaneously detected successfully in one reaction. However, the potential of the LDR/melting curve assay may be far higher than this. One of the most useful aspects of LDR is that sets of probes for a number of mutation sites can be multiplexed together, enabling several mutations to be detected simultaneously. For example, in a study investigating the mutations which cause cystic fibrosis, LDR was able to identify 30 mutations in a single reaction[16]. In general, real-time PCR instruments have at least four measurement channels for different fluorescence signal detection; in the same measurement channel, melting temperatures of ligation products could be designed to range from 60℃-80℃, with 3-4℃ intervals. Therefore, a single four-color assay could be used to detect more than 20 mutations simultaneously. Compare to the other mutation detection methods based on melting analysis such as high resolution melting analysis (HRMA), our strategy has the advantage of high throughput while HRMA detected only one mutation in one reaction generally[23].

In order to simplify and ensure the success of establishing this method, the LDR/melting curve assay of genomic DNA used the PCR and employed the resulting PCR products as LDR templates. The disadvantage of using PCR post-analysis is that it may limit the use of new technologies in clinical sample detection. Previous studies utilizing PCR and LDR in a single tube for amplification show that PCR and LDR could perform in a continuous process[24]. In future studies, we aim to design a reaction system whereby PCR, LDR and melting curve assay occur in a single tube, thereby optimizing the multiplex mutation detection system for clinical applications.

Conclusions

In summary, this novel LDR with melting curve assay represents a suitable method for multiplex detection of genetic mutations, and provides a new analysis strategy for LDR product. This method is an important advance in mutation detection as it retains the high-fidelity previously shown with LDR and increases the potential for multiplex detection in a relatively simple technology platform. In view of these advantages, this mutation detection strategy is expected to provide an intrinsically robust and specific mutation detection platform for large-scale screening the carrier of inheritance disease and primary diagnosis of genetics disease before sequencing or other tests.

Acknowledgements

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