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RNA SNP Detection Method With Improved Specificity Based on Dual-competitive-padlock-probe*

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Abstract **Objective** The detection of RNA single nucleotide polymorphism (SNP) is of great importance due to their association with protein expression related to various diseases and drug responses. At present, splintR ligase-assisted methods are important approaches for RNA direct detection, but its specificity will be limited when the fidelity of ligases is not ideal. The aim of this study was to create a method to improve the specificity of splintR ligase for RNA detection. **Methods** In this study, a dual-competitive-padlock-probe (DCPLP) assay without the need for additional enzymes or reactions is proposed to improve specificity of splintR ligase ligation. To verify the method, we employed dual competitive padlock probe-mediated rolling circle amplification (DCPLP-RCA) to genotype the *CYP2C9* gene. **Results** The specificity was well improved through the competition and strand displacement of dual padlock probe, with an 83.26% reduction in nonspecific signal. By detecting synthetic RNA samples, the method demonstrated a dynamic detection range of 10 pmol/L−1 nmol/L. Furthermore, clinical samples were applied to the method to evaluate its performance, and the genotyping results were consistent with those obtained using the qPCR method. **Conclusion** This study has successfully established a highly specific direct RNA SNP detection method, and provided a novel avenue for accurate identification of various types of RNAs.

Key words RNA, single nucleotide polymorphism, genotyping, rolling circle amplification, dual padlock probe
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Single nucleotide polymorphisms (SNPs) represent one of the most prevalent genetic variations in humans, with a significant number of SNPs directly or indirectly associated with interindividual differences in phenotype, susceptibility to diseases, and drug responses[1-5]. The detection of SNPs plays an indispensable role in identifying disease-causing genes, predicting drug responses, and personalizing treatment regimens[6-10]. Existing SNP detection technologies mostly target DNA as the testing sample. However, transcription errors, with an error rate of around 10^{-6}, can occur at any position within any gene, affecting various aspects of protein structure and function[11]. Consequently, RNA provides a more direct and precise reflection of protein expression than DNA. It is meaningful to conduct detection at the RNA level for studies on SNP-phenotype association like genotyping for personalized drug guidance[12]. Furthermore, the emergence of the corona virus disease 2019 (COVID-19) has also highlighted the importance of RNA-related research, such as severe

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acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA expression mechanism exploration[13], SARS-CoV-2 RNA detection[14-15], and the rapid development of mRNA vaccine[16].

Current RNA detection technologies primarily include reverse transcription quantitative PCR (RT-qPCR) [17-18], and RNA sequencing (RNA-seq) [19-20]. However, these methods require instruments with high thermal cycling performance, which can be costly and unsuitable for resource-limited regions. Additionally, there are some isothermal RNA detection technologies with lower temperature requirements, such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) [21-23], and padlock probe-mediated rolling circle amplification (PLP-RCA) [24-26]. However, these methods usually require conversion of RNA to cDNA through reverse transcription, which may introduce base errors and lost its base modification information[27].

To enable direct detection of RNA, enzymes with the capability to act on RNA have been explored[28-29]. For instance, RNA templated DNA ligation activity of T4 DNA ligase has been discovered, but the low ligation efficiency on RNA limits its utility in RNA SNP detection[30]. In recent years, splintR DNA ligase has been discovered to be capable of RNA templated DNA ligation with high efficiency, demonstrating the potential for direct RNA detection. The splintR-assisted ligation has been combined with PLP-RCA for direct RNA detection, which has been extensively developed and applied in the detection of miRNA[31-32], mRNA[33], as well as SARS-CoV-2 virus RNA[34]. The detection efficiency is significantly improved by combining with techniques such as time-gated Forster resonance energy transfer (TG-FRET)[31], and hybridization chain reaction (HCR)[34]. However, the fidelity of splintR ligase for certain base is not ideal, a research shows that the splintR ligase demonstrates a fidelity of 92% for the 5' A base on RNA, whereas the fidelity drops significantly to only 34% for the 3' C base[35]. Its ability to specifically recognize single-base differences in certain sequences is challenging, thus limiting its application in RNA SNP detection when the mutation site is a low-fidelity base.

To enhance the specificity of the splintR-assisted detection method, strategies combining advantages of different amplification techniques have been proposed. For example, Zhou *et al*.[36] established a highly sensitive and specific RNA detection method by combining splintR ligase ligation with PCR amplification and CRISPR/Cas12a cleavage. However, this approach requires the integration of other methods to improve specificity and does not directly address the specificity of splintR ligase ligation. To enhance the specificity of splintR ligase ligation, an invader padlock probe method that combines with the invader assay principle has been proposed[37]. With the invader padlock probe, the ligation specificity has been significantly improved. However, this approach requires the introduction of an extra enzyme and invader padlock probe activation step.

Traditional competitive strategies involve designing dual-fluorescent probes or dual-primers, typically used in PCR reactions to enhance specificity. Amplification of separated ligation-dependent probes (ASLP) technology designed two probes that selectively pair with different alleles to obtain specific signals. The probe can ligate with adjacent primer when fully matching the target. The resulting ligation products are then separated and undergo PCR amplification[38]. Universal probe-based intermediate primer-triggered qPCR (UPIP-qPCR) method has been employed to improve the accuracy of genotyping, with two primers including different probe binding sequences that compete with each other[39]. Combination and competition probe (Com probe) assay designed a non-fluorescently labeled probe, pairing with the wild-type target, to compete with the fluorescently labeled probe that pairs with the mutant target. The approach has been used to enhance the specificity of SARS-CoV-2 variant detection[40]. Here, we applied the competitive strategy to the padlock probe design and proposed a dual-competitive-padlock-probe (DCPLP) assay that can improve splintR ligation specificity without the need for additional enzymes and reactions. Due to the competition of dual-padlock-probe binding to the target, nonspecific padlock probe-target complex will be displaced by the perfectly matched padlock probe-target complex, thereby improving the ligation specificity. To validate the effectiveness of this method, we employed dual competitive padlock probe-mediated rolling circle amplification (DCPLP-RCA) for RNA SNP genotyping assay and selected the *CYP2C9* gene, which is associated with efficacy

and adverse reactions of multiple drugs[41-42], as the detection target. By combining rolling circle amplification (RCA) and fluorescence quenching (FQ) probes, the circularized padlock probe can be amplified, and the signal can be collected in real-time. Compared to the traditional single-padlock-probe system, the DCPLP assay can improve specificity through competition and strand displacement. Moreover, the concordance between DCPLP-RCA and qPCR genotyping results suggests an application potential of our method for highly specific direct RNA SNP detection.

1 Materials and methods

1.1 Materials and instruments

The splintR ligase used in this study was purchased from New England BioLabs Inc. (USA). Red blood cell lysis solution and dNTPs were obtained from Sangon Biochemistry Co., Ltd. (Shanghai, China). RNA-easy Isolation Reagent, phi29 DNA polymerase, Taq Pro HS Universal Probe Master Mix, and ROX (50×) were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). BSA was obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Agarose, DNA marker, YeaRed nucleic acid dye (10 000×), and 6×loading buffer were acquired from Yeasen Biotechnology Co., Ltd. (Shanghai, China). *Exo* I was sourced from Takara Bio Inc. (Japan). TaqMan™ Genotyping Master Mix, GeneJET Genomic DNA Purification Kit, ProFlex thermal cycling PCR instrument, Applied Biosystems™ 7500 Real-Time PCR System, Applied Biosystems™ QuantStudio7 Real-Time PCR System, and NanoDrop™ 2000 Spectrophotometer were purchased from Thermo Fisher Scientific Biotechnology Co., Ltd. (USA). The Bio-Rad Gel Doc XR+ gel imaging system was procured from Bio-Rad Laboratories, Inc. (USA).

1.2 Oligonucleotide design

Wild and mutant RNA target were designed with reference to the rs1057910 mutation site of *CYP2C9* gene. The dual padlock probe sequence consists of 3 functional regions: the terminal probe arms, the primer binding region, and the FQ probe region. The terminal probe arms can bind to the RNA target, with the 3' end pairing with the mutation site. The primer binding region can bind to the primer (P) to trigger rolling circle amplification. After the formation of rolling circle products, the FQ probe region can bind to the FQ probe to generate signals. The dual padlock probes are named wild padlock probe (WPLP) and mutant padlock probe (MPLP). Their probe arms are designed to fully match the wild-type target and mutant target, respectively. After replication, their FQ probe regions bind to the wild fluorescence probe (WF) and mutant fluorescence probe (MF) to differentiate the signals of different targets. The fluorescence probes are modified with 6-carboxyfluorescein (FAM) and 2', 7'-dimethoxy-4', 5'-dichloro-6-carboxyfluorescein (VIC) fluorescent groups, while the quenching probe (Q) is modified with BHQ1 quenching group. When they bind to the rolling circle products, the fluorescent group and the quenching group are close to each other, resulting in fluorescence signal reduction. All oligonucleotide sequences used in this study were synthesized by Sangon Biochemistry Co., Ltd. (Shanghai, China), as listed in Table S1.

The design optimization results of the padlock probe are shown in Figure S1. During the experimental process, we obtained highly specific padlock probes by varying the length of detection arm. We designed two wild-type padlock probes (WPLP* and WPLP), with their sequences shown in Table S1. The 5' detection arm lengths of WPLP* and WPLP were 13 and 12 nucleotides, respectively, while the 3' detection arms were both 12 nucleotides long, with the remaining sequences being identical. WPLP* and WPLP probes were mixed with the MPLP probe and used for detecting three different genotypes (WW, WM, and MM) samples. When one terminal arm (5') of the padlock probe is longer and the other terminal arm (3') has a nucleotide difference, the binding energy between the padlock probe and the target is determined by the longer terminal arm, resulting in stronger binding and poor specificity. Therefore, it is important to ensure comparable lengths of the detection arms at both ends when designing padlock probes so that when there is a nucleotide difference at the 3' end, the difference in binding energy with the target is significant, leading to better recognition.

1.3 Preparation of circular DNA

Equal volumes of wild and mutant RNA target (1 μmol/L) and padlock probe W/MPLP (2 μmol/L) were added to a microcentrifuge tube and vortexed thoroughly. The tube was heated to 80℃ for 5 min and then slowly cooled to 25℃. Next, splintR ligase

(25 U) and 10×splintR buffer (1×) were added to the reaction mixture, which was mixed by shaking and centrifuged before being incubated at 25℃ for 2 h. The reaction was terminated by heating at 65℃ for 20 min. After the previous step, 50 U *Exo* I and 10× *Exo* I buffer (1×) were added to the microcentrifuge tube containing the reaction product, and the mixture was mixed by shaking and incubated at 37℃ for 30 min. The enzymatic activity was terminated by incubating the mixture at 80℃ for 15 min. The resulting products of each step were characterized by agarose gel electrophoresis (Figure S2). The resultant circular DNA was stored at −20℃ for future use.

1.4 Real−time monitoring of the RCA reaction

Circular DNA was used as the template for the RCA reaction. The reaction mixture consisted of primer P (1 μmol/L), FQ probe (2 μmol/L, containing 2 μmol/L of fluorescence probe WF or MF, and 2 μmol/L of quencher probe Q), BSA (2 g/L), dNTPs (1 mmol/L each), ROX (2.5×), phi29 buffer (1×), and phi29 DNA polymerase (10 U), with the total volume adjusted to 20 μl using ddH_2O. The reaction was carried out on a 7500 Real-Time PCR System, with the following conditions: 30℃ for 2 h, for a total of 30 cycles. The fluorescence signal was continuously recorded in real time by the qPCR instrument.

1.5 Formula processing of fluorescence signal

The processing of fluorescence signals in the experiment is as follows: the fluorescence intensity is collected by QuantStudio7 Real-Time PCR System, and the fluorescence change value represents the variation in fluorescence intensity during the experimental process.

Here, we begin by normalizing the fluorescence intensity by subtracting the fluorescence intensity of the reference dye ROX, resulting in a normalized reporter. The calculation formula is as follows:

$$Rn = FI/FI_{ROX} \quad (1)$$

where Rn is the normalized reporter, FI is the fluorescence intensity in the FAM or VIC channel, and FI_{ROX} is the fluorescence intensity of the reference dye ROX.

Subsequently, to obtain the fluorescence change value, we subtracted the normalized initial value from the endpoint value, as described by the following formula:

$$\Delta Rn = Rn_i - Rn_e \quad (2)$$

where ΔRn is the normalized fluorescence change value, Rn_i represents the normalized initial fluorescence reporting group value, and Rn_e represents the normalized endpoint fluorescence reporting group value.

The consumption rate of FQ probe signifies the proportion of the consumed quantity of FQ probe during the experimental process relative to the total input of FQ probe. Here, we employ fluorescence intensity values as a representation of the quantity of FQ probe. Hence, the consumption rate of the FQ probe is obtained through the following formula:

$$CR = \left[\left(FI_{max} - FI_{min}\right)/FI_{max}\right] \times 100\% \quad (3)$$

where CR denotes the consumption rate of the FQ probe, FI_{max} represents the maximum fluorescence intensity value, and FI_{min} represents the minimum fluorescence intensity value.

1.6 Detection of clinical samples

Twelve clinical blood samples were collected from the second affiliated hospital of Soochow University. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University (JD-LK2023007-I01). Written informed consents were obtained from each of the involved individuals.

Each blood sample (1.2 ml) was divided into two parts. One part (1 ml) was treated with red blood cell lysis solution to remove red blood cells and collect white blood cells. Subsequently, total RNA was extracted using the RNA-easy Isolation Reagent (TRIzol) and dissolved in 10 μl of nuclease-free water. Within the RNA solution, 1.5 μl of a mixture of WPLP and MPLP (WMPLP, 10 μmol/L) was added and heated at 95℃ for 2 min before slowly cooling to room temperature. Next, 1.5 μl of 10×splintR buffer and 25 U of splintR ligase were added to the previous reaction mixture, followed by incubation at 25℃ for 2 h and inactivation at 65℃ for 20 min. Afterward, 10 U of *Exo* I, 2 μl of 10×*Exo* I buffer, and ddH_2O were added to the ligation reaction mixture to reach a final volume of 20 μl. The mixture was gently shaken and digested at 37℃ for 30 min, followed by inactivation at 80℃ for 15 min, resulting in a circular template solution. Finally, 7 μl of the circular template obtained previously was used in the RCA reaction system, which included primer P (200 nmol/L), FQ probe (200 nmol/L), BSA (2 g/L), dNTPs (200 μmol/L each), ROX (2.5×), phi29 buffer (1×), and phi29 DNA polymerase (5 U). The reaction volume was adjusted

to 20 μl with ddH_2O, and the reaction was performed on a 7500 Real-Time PCR System. The reaction conditions were set at 30℃ for 2.5 h. For the other part (200 μl), DNA was extracted using the GeneJET Genomic DNA Purification Kit and eluted with 50 μl of nuclease-free water. Then, 9 μl of the eluted DNA was mixed with 1 μl of TaqMan™ Genotyping Master Mix and 10 μl of Taq Pro HS Universal Probe Master Mix for qPCR genotyping. The reaction was carried out on a QuantStudio7 Real-Time PCR System, with an initial incubation at 95℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 1 min. Finally, the reaction mixture was incubated at 60℃ for 30 s to read the data.

2 Results and discussion

2.1 The working principle of DCPLP−RCA

The working principle of RNA SNP genotyping method based on dual competitive padlock probe-mediated rolling circle amplification (DCPLP-RCA) is shown in Figure 1.

Figure 1a illustrates the experimental process of DCPLP-RCA for RNA SNP genotyping. Firstly, the RNA target is hybridized with the padlock probe, resulting in the formation of four kinds of padlock probe-target complexes through random pairing. Then, a ligation reaction is performed using splintR ligase, forming the circular DNA. However, due to the suboptimal fidelity of splintR ligase, nonspecific ligations can occur during this process. The circular DNA can serve as a template for subsequent RCA with FQ probes. The two circular DNA templates contain distinct probe binding regions that can bind to their respective FQ probes, enabling real-time monitoring of the amplification process. When fluorescence (F) probe and quencher (Q) probe come close to each other upon binding to the target, the fluorescence of F probe is quenched by Q probe, resulting in a decrease in fluorescence intensity. The two alleles are represented by two fluorescence channel signals (VIC, FAM) respectively. As different genotypes possess different alleles, the fluorescence signals obtained from the interaction with the padlock probes exhibit variation. By comparing and analyzing the signals from the two fluorescence channels, the different genotypes can be determined.

Figure 1b shows the mechanism of DCPLP to inhibit nonspecific signal. There is a comparison of random pairing process with single and dual padlock probe. The conventional SNP detection system typically employs single padlock probe, where only one MPLP that fully matches the mutant target is introduced. In the single-padlock-probe system, when the wild target is detected, it can only form an incompletely matched padlock probe-target complex with MPLP. Due to the suboptimal fidelity of splintR ligase for terminal ligation, there can be a significant amount of nonspecific ligation. In contrast, the dual-padlock-probe system of DCPLP-RCA incorporates two padlock probes that fully match two different alleles. When wild target is detected, it can respectively form two distinct padlock probe-target complexes with the two padlock probes, which exist in dynamic equilibrium. As the binding free energy is lower and the resulting duplex is more stable for padlock probe that perfectly match the targets, nonspecific padlock probe-target complex will be displaced by the perfectly matched padlock probe-target complex, inhibiting the formation of incompletely matched complexes. Consequently, subsequent nonspecific ligation and amplification are suppressed, reducing the impact of the suboptimal fidelity of splintR ligase for terminal ligation. Therefore, compared to the single-padlock-probe system, the dual-padlock-probe system exhibits inhibited nonspecific signals, thus enhancing the specificity of RNA SNP detection.

2.2 The optimization of DCPLP−RCA

To achieve more desirable experimental results, a step-by-step optimization of the DCPLP-RCA reaction parameters was conducted. Adjustment and optimization were performed for parameters such as primer P, dNTP, phi29 polymerase, and FQ probe. While adjusting the concentration of one parameter, the remaining experimental conditions were kept constant and in excess. The optimization results of the experimental conditions are shown in Figure 2. Figure 2a demonstrates that as the concentration of primer P increases, the fluorescence change ΔRn initially rises and then decreases in the FAM channel. The influence of primer concentration on ΔRn is mainly related to the ratio of circular template and primer. When the primer concentration is too low, the RCA product tends to be longer but with fewer number of reaction units, resulting in a low product formation rate and a

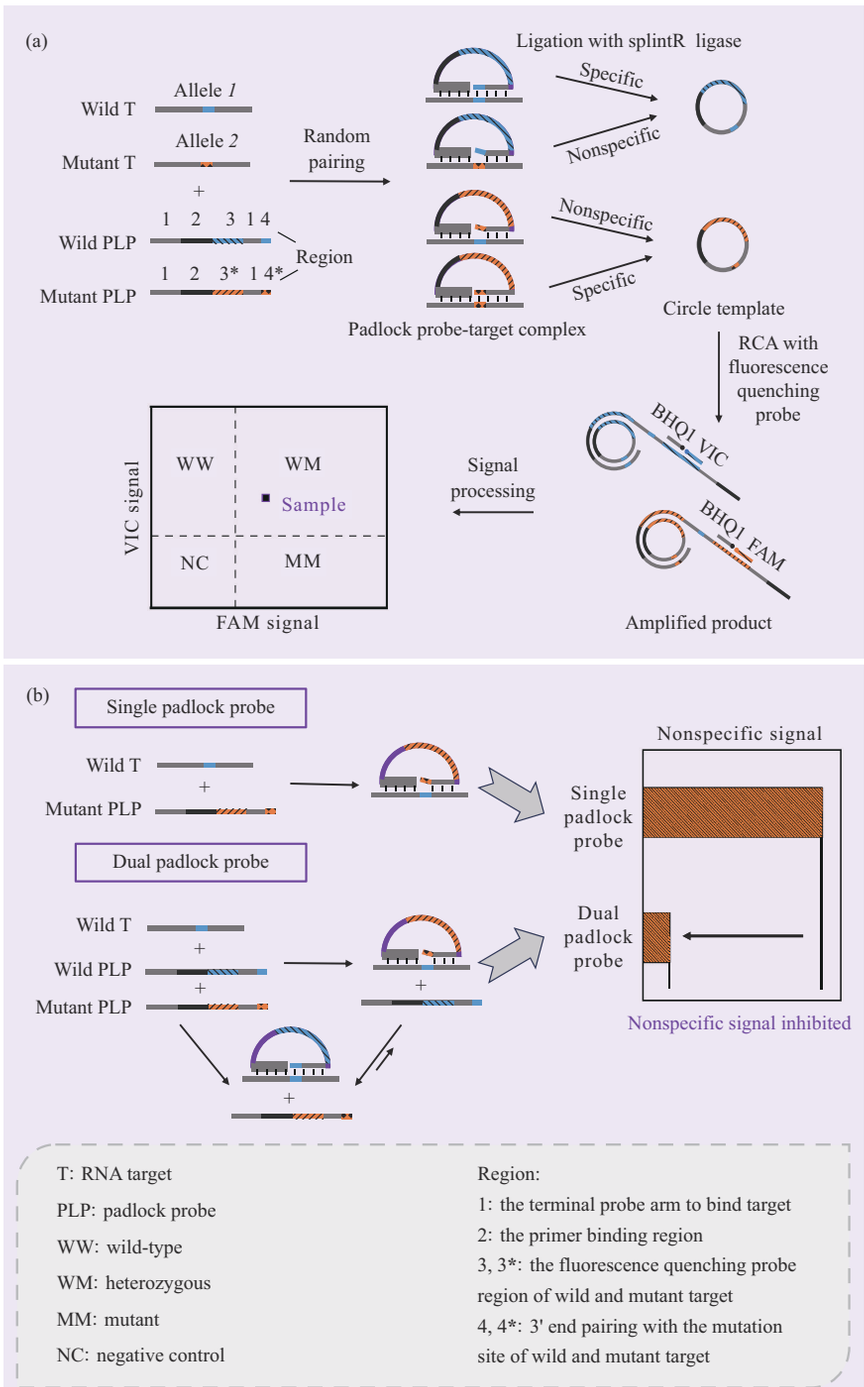

Fig. 1　The working principle of RNA SNP genotyping method based on dual competitive padlock probe−mediated rolling circle amplification (DCPLP−RCA)

(a) The schematic diagram of DCPLP-RCA for RNA SNP genotyping. Firstly, the RNA target forms padlock probe-target complex with padlock probe through random pairing. Subsequently, splintR ligase is added to join the padlock probes within this complex, resulting in circular DNA. Then, RCA is performed using the circular DNA as a template, while simultaneously monitoring the fluorescence signals in real time. Finally, the obtained fluorescence signals are normalized, processed, and plotted to generate a genotype distribution map. (b) The mechanism of DCPLP to inhibit nonspecific signal. In the dual-padlock-probe system, nonspecific padlock probe-target complex will be displaced by the perfectly matched padlock probe-target complex, inhibiting the formation of nonspecific complexes during the random pairing process.

low Δ*R*n. When the primer concentration is too high, more attachment sites for the enzyme are available, leading to an increased RCA reaction units and the synthesis of more products, but with shorter lengths. Many products may not have complete binding regions with the FQ probes, resulting in the decrease of Δ*R*n. When the primer concentration is moderate, the RCA products reach a balance in terms of quantity and length, resulting in the maximum Δ*R*n. The maximum Δ*R*n is observed at a P concentration of 200 nmol/L, indicating the optimal concentration for primer P in the experiment. As shown in Figure 2b, as the dNTP concentration increased, Δ*R*n initially increased and then decreased. The maximum Δ*R*n is obtained when the dNTP concentration is 200 μmol/L. A further increase in dNTP concentration leads to a decrease in fluorescence change. Therefore, 200 μmol/L was determined to be the optimal concentration for dNTPs in the experiment. Figure 2c illustrates the impact of phi29 DNA polymerase on the reaction. With an increasing amount of phi29 DNA polymerase, the fluorescence change gradually increases and eventually reaches a plateau. When the enzyme amount reaches 5 U, further increase does not significantly affect Δ*R*n. Hence, 5 U was chosen as the most suitable amount of phi29 DNA polymerase. Figure 2d illustrates the impact of different FQ probe concentrations on Δ*R*n as well as the consumption rate at each concentration. As the FQ probe concentration increases, both Δ*R*n and the consumption rate experience a sharp increase and reach their maximum at an FQ concentration of 200 nmol/L. However, further increasing the FQ probe concentration to 2 μmol/L results in a low consumption rate of only 4.63%, indicating extremely low utilization efficiency. Therefore, 200 nmol/L was chosen as the optimal concentration for the FQ probe in subsequent experiments.

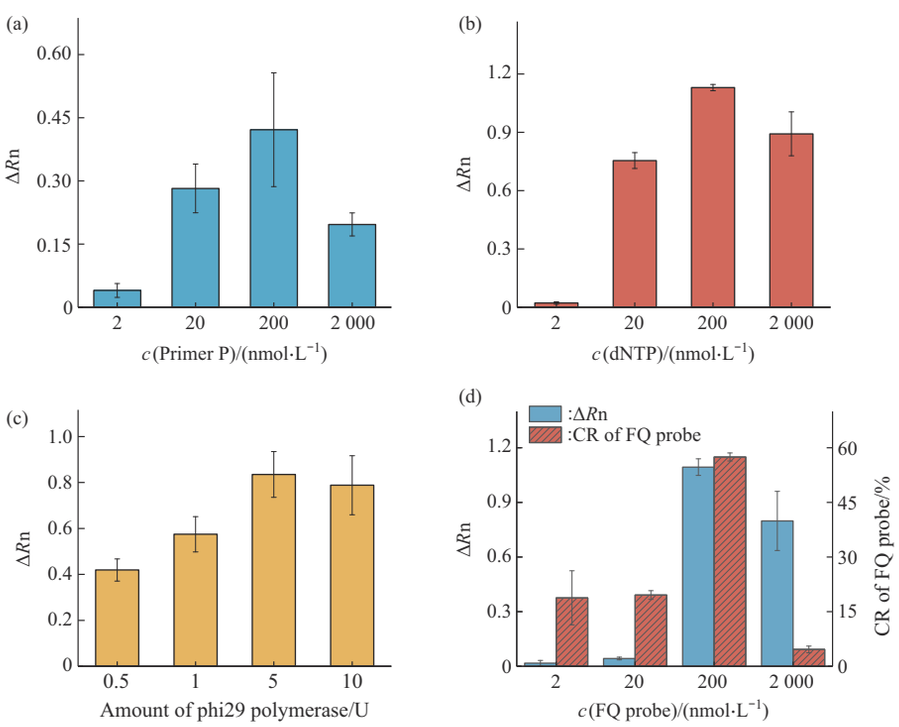

Fig. 2 The optimization of DCPLP−RCA

(a) The impact of the primer P concentration on Δ*R*n. (b) The effects of dNTP concentration on Δ*R*n. (c) The impact of phi29 polymerase quantity on Δ*R*n. (d) The influence of different FQ probe concentrations on Δ*R*n and the consumption rate (CR) of FQ probe.

2.3 The feasibility validation of DCPLP−RCA

To validate the feasibility of applying the DCPLP-RCA method for RNA SNP genotyping, synthetic RNA wild-type target and mutant target were combined to create three genotypic samples: wild-type (WW), heterozygous (WM), and mutant (MM). The genotyping results, shown in Figure 3, represent the FAM channel for the mutant signal and

the VIC channel for the wild-type signal. In Figure 3a, the FAM channel represents the mutant signal. WW samples without mutant target exhibit minimal signal reduction, WM samples containing 50% of mutant target show significant signal reduction, and MM samples containing 100% of mutant target display the highest signal reduction. Figure 3b represents the real-time signal changes in the VIC channel, representing the wild-type signal. MM samples without wild target show minimal signal reduction, WM samples containing 50% of wild target display substantial signal reduction, and WW samples containing 100% of wild target exhibit the highest signal reduction. By plotting the FAM and VIC signal changes within 30 cycles, as shown in Figure 3c, it becomes evident that the data points corresponding to the WW, WM, and MM genotypes can be clearly distinguished. The obtained results were subjected to linear fitting, and the theoretical genotyping thresholds were calculated based on the slope in conjunction with Δ*R*n. Two thresholds are obtained by adding the Δ*R*n (FAM) of the WW and the Δ*R*n (VIC) of MM, along with 10 times the standard deviation of the negative control group, into the linear equation of the WM and then selecting the larger value (Figure S3).

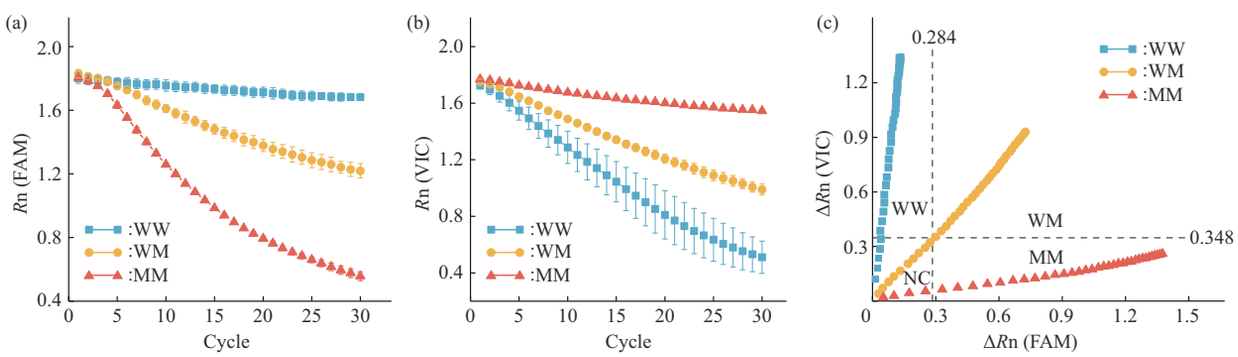

Fig. 3 The feasibility validation of DCPLP−RCA

(a) Real-time fluorescence changes of the three genotypes in the FAM channel, which represent mutation signals. (b) Real-time fluorescence changes of the three genotypes in the VIC channel, which represent wild signals. (c) Genotyping results obtained by using the Δ*R*n in the FAM and VIC channels as the *x* and *y* coordinates, respectively. WW: wild-type sample, WM: heterozygous sample, MM: mutant sample.

2.4 The specificity of the DCPLP−RCA

To validate the specificity of the experimental method, we simulated wild-type (WW) and mutant-type (MM) samples with synthetic wild and mutant RNA templates. These samples were then combined with two specific padlock probes (WPLP and MPLP) and their mixed probe (WMPLP) for ligation and RCA reaction. The real-time changes and comparison of fluorescence signals obtained in the experiment are shown in Figure 4. As depicted in Figure 4a−c, when the 3 probes were applied to the wild-type sample (WW), WPLP and WMPLP exhibited weak signals in the FAM channel and strong signals in the VIC channel. MPLP showed low signal intensity in both channels, indicating that the splintR ligase has good fidelity for the detection of the “A” nucleotide in the wild-type sample. As depicted in Figure 4d−f, when detecting the mutant sample (MM), MPLP and WMPLP showed strong signals in the FAM channel and weak signals in the VIC channel, which is consistent with expectations. However, WPLP exhibited a relatively strong signal in the VIC channel, indicating low fidelity of the splintR ligase for the “C” nucleotide in the mutant sample, resulting in nonspecific ligation and amplification. By comparing the Δ*R*n values of WMPLP and WPLP in the VIC channel, it can be observed that when using splintR ligase to detect nucleotides that cannot be specifically recognized, WMPLP not only effectively expresses specific signals but also inhibits nonspecific signals. WMPLP showed a high normalized signal expression level for both the WW and MM samples, with values of 0.939 and 0.734, respectively. When detecting the mutant sample (MM), the nonspecific signal Δ*R*n value has an 83.26% reduction, decreasing from 1.290 (WPLP) to 0.216 (WMPLP), resulting in an improvement of specificity (Table S2). Compared to other RNA detection methods, our approach is characterized by high specificity, allowing for direct detection of SNP on RNA (Table S3).

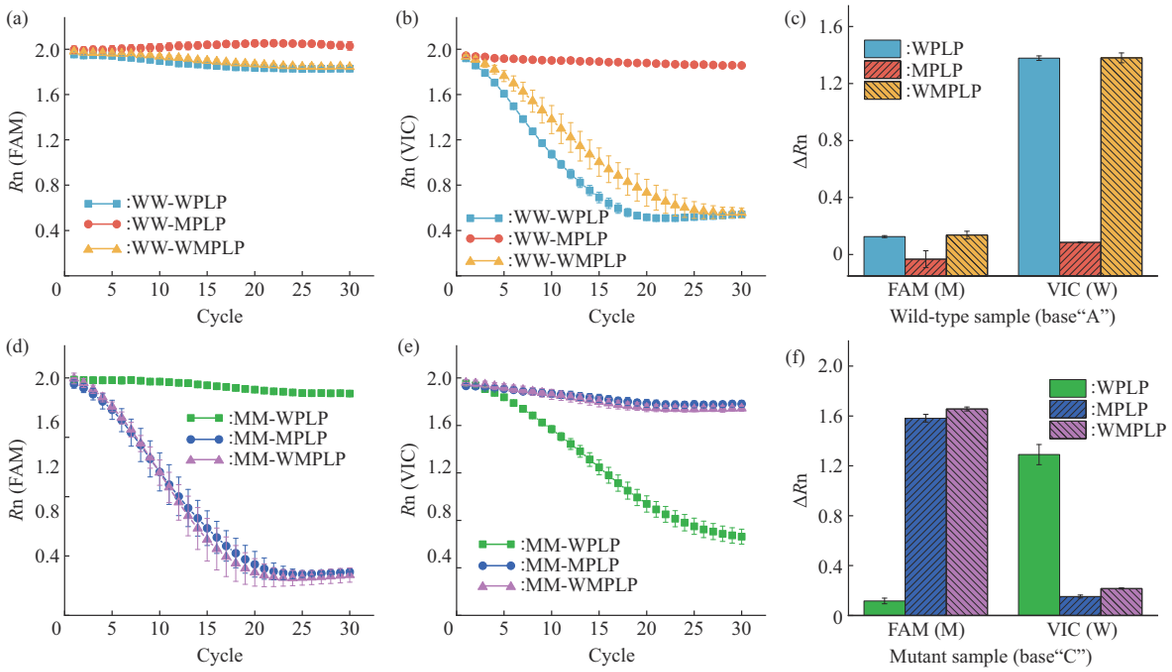

Fig. 4　The specificity of DCPLP−RCA

(a) The FAM fluorescence changes of wild-type sample (WW) when reacting with three padlock probes (WPLP, MPLP, and WMPLP). (b) The VIC fluorescence changes of wild-type sample (WW) when reacting with three padlock probes. (c) The comparison of fluorescence signal changes obtained from the reaction with the wild-type sample. (d) The FAM fluorescence changes of mutant samples (MM) when reacting with three padlock probes. (e) The VIC fluorescence changes of mutant samples (MM) when reacting with three padlock probes. (f) The comparison of fluorescence signal changes obtained from the reaction with the mutant sample. WPLP: specific padlock probe for wild-type sample, MPLP: specific padlock probe for mutant sample, WMPLP: mixed padlock probe of WPLP and MPLP.

2.5　The sensitivity of the DCPLP−RCA

To evaluate the performance of the DCPLP-RCA method, we measured the fluorescence signal changes of mutant targets with different concentrations under optimized conditions. As shown in Figure 5a, the target can be detected within the concentration range of 10 pmol/L−1 nmol/L. A fitting analysis is performed within the range of 10−100 pmol/L, as shown in Figure 5b, and a good linear relationship between Δ*R*n and the target concentration is obtained. According to the 3σ principle, the detection limit is determined to be 6 pmol/L. The sensitivity of the method is not outstanding, possibly due to the limited amplification efficiency of RCA, a linear amplification reaction. In order to improve the detection sensitivity, endonuclide can be used to cut RCA products into repeated fragments for secondary netlike rolling circle amplification (NRCA), with a detection limit of 0.1 fmol/L[43] or signal accumulation amplification combined with hybridization chain reaction (HCR), with a detection limit of 0.316 pmol/L[44].

2.6　Mutation frequency detection of DCPLP−RCA

To assess the ability of DCPLP-RCA to detect different mutation frequencies, wild-type templates and mutant templates were mixed at various proportions to obtain samples with mutation frequencies of 20%, 40%, 60%, and 80% of the total template concentration. These samples underwent ligation reactions and RCA, and the detection effectiveness for mutation frequency was observed by monitoring changes in fluorescence signals. The genotyping results are presented in Figure 6. It illustrates the detection outcomes for samples with different mutation frequencies, where each mutation frequency data point is clearly distinguishable. The mutation frequency shows a good linear correlation with both normalized fluorescence change value (Δ*R*n) in FAM and VIC channels.

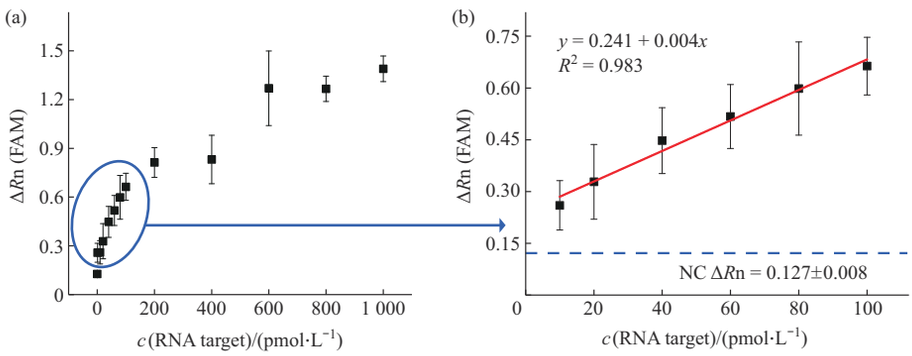

Fig. 5　The sensitivity of DCPLP−RCA

(a) The relationship between Δ*R*n and target concentrations in the 10 pmol/L−1 nmol/L range. (b) The linear relationship between Δ*R*n and target concentrations in the range of 10−100 pmol/L.

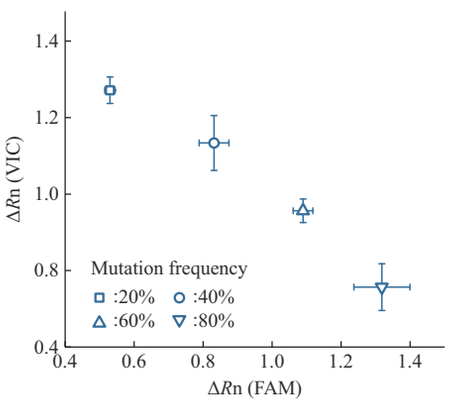

Fig. 6　The distribution results of DCPLP−RCA method for detecting samples with different mutation frequencies

2.7　Clinical sample genotyping analysis with DCPLP−RCA

Considering that the DCPLP-RCA method allows for direct detection of RNA SNP with high specificity, we plan to test the feasibility of this method for genotyping in clinical samples. CYP2C9 is an important drug-metabolizing enzyme in the human body, and its genetic polymorphisms are associated with the metabolism of various drugs, including warfarin, celecoxib, and duloxetine[41-42]. Conducting CYP2C9 genotyping on patients before administering these medications enables physicians to personalize and precisely prescribe medication based on the patient's genotype, thereby reducing overmedication and adverse reactions[45-46]. Twelve fresh blood samples were collected for detection of the CYP2C9*3 genotype. The genotyping of the same samples was performed using both the DCPLP-RCA method and qPCR method. In Figure 7, the results from both methods showed that only sample 8 was heterozygous, while all other 11 samples were wild type. Comparisons indicated that our method was consistent with the qPCR method in the experimental validation of clinical samples, confirming the

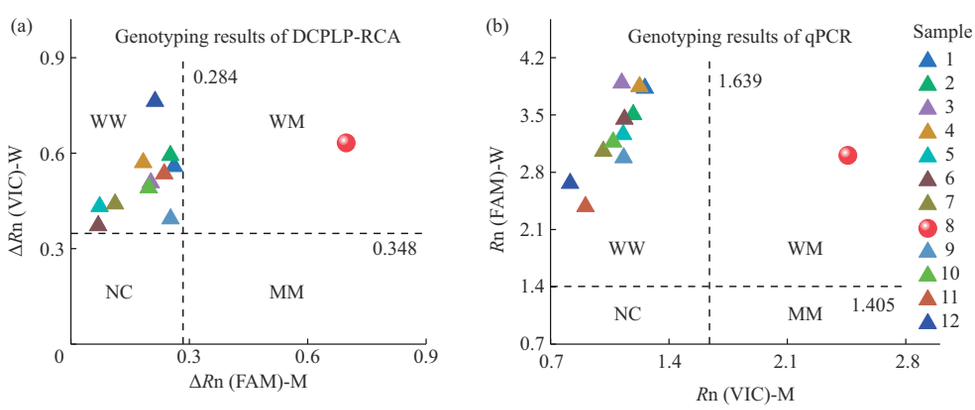

Fig. 7　Genotyping analysis results in clinical samples for comparison of the DCPLP−RCA method and qPCR method

(a) Genotyping results of clinical samples using the DCPLP-RCA method. The Δ*R*n values have already been corrected for background noise from the negative control group. (b) Genotyping results of clinical samples using the qPCR method. The two thresholds of qPCR were determined by using synthetic plasmid to simulate three genotypic samples like DCPLP-RCA. WW: wild-type sample, WM: heterozygous sample, MM: mutant sample.

feasibility of applying the DCPLP-RCA method for direct RNA SNP genotyping in clinical samples.

3 Conclusion

In summary, we have proposed a DCPLP-RCA method for direct RNA SNP genotyping by combining the DCPLP assay, which enhances the specificity of splintR ligation, with the RCA reaction. The results demonstrate that the nonspecific signal is reduced by 83.26% through dual-padlock-probe competition and strand displacement, thus confirming that the DCPLP assay indeed improves the specificity of splintR ligation. The method can detect the target at the concentration of 10 pmol/L−1 nmol/L and has a good linear relationship in the range of 10−100 pmol/L. The method was also applied to genotype *CYP2C9* gene in 12 clinical samples, yielding 11 cases of wild type and 1 case of heterozygous, which is consistent with the results obtained using the qPCR method. Hence, the DCPLP-RCA provides a potential platform for highly specific direct RNA SNP detection, providing the possibility for genotyping for personalized drug guidance. With DCPLP assay, various types of RNAs can be specifically identified. The DCPLP assay can be applied in different combinations of ligase ligation and amplification reaction, and it can also be employed for DNA analysis.

Supplementary Available online (http://www.pibb.ac.cn, http://www.cnki.net):
PIBB_20240101_Figure S1.pdf
PIBB_20240101_Figure S2.pdf
PIBB_20240101_Figure S3.pdf
PIBB_20240101_Table S1.pdf
PIBB_20240101_Table S2.pdf
PIBB_20240101_Table S3.pdf

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基于双竞争挂锁探针改进RNA SNP检测特异性的方法*

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摘要　目的　RNA的单核苷酸多态性（single nucleotide polymorphism，SNP）与多种疾病和药物反应相关的蛋白质表达有关，因此对其检测具有重要意义。目前，splintR连接酶辅助方法是RNA直接检测的重要方法，但当连接酶的保真度不理想时，该方法的特异性将受到限制。本研究的目的是创建一种提高splintR连接酶检测RNA特异性的方法。**方法**　本研究提出了一种双竞争挂锁探针（dual-competitive-padlock-probe，DCPLP）检测方法，不需要额外的酶或反应，可提高splintR连接酶的特异性。为了验证该方法，采用双竞争挂锁探针介导的滚环扩增（rolling circle amplification，RCA）对*CYP2C9*基因进行RNA SNP基因分型。**结果**　通过双挂锁探针的竞争和链替换，SNP检测的特异性得到了很好的提高，非特异性信号降低了83.26%。通过引入合成RNA样品，实现了10 pmol/L~1 nmol/L的动态检测范围。并将临床样本应用于该方法进行性能评价，结果与qPCR方法的基因分型结果一致。**结论**　本研究成功建立了一种高特异性的RNA SNP直接检测方法，为准确鉴定各类RNA提供了新的途径。

关键词　RNA，单核苷酸多态性，基因分型，滚环扩增，双挂锁探针
中图分类号　Q522，O65　　**DOI**：10.16476/j.pibb.2024.0101

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