Research Square

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Optimization of two-step asymmetric PCR protocol to prepare single-strand DNA for aptamer screening

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Abstract

Background

Aptamers are promising molecular recognition elements. During aptamer screening using the systematic evolution of ligands by exponential enrichment (SELEX) technology, the preparation of single-stranded DNA (ssDNA) is crucial. Asymmetric PCR (A-PCR) is commonly used for ssDNA generation. Due to the complexity of ssDNA library templates and the variability of PCR reagents, optimizing A-PCR is essential to obtain an enriched ssDNA library with high yield and purity.

Methods and Results

In this study, the general conditions were initially optimized through single-factor experiments in symmetric PCR. The optimal conditions were 4.8 fmol/μL ssDNA template, 20 μM dNTP, 80 nM forward and reverse primers, 2 U Taq DNA polymerase, 1.5 mM Mg^{2+}, an annealing temperature of 62°C, and 14 PCR amplification cycles. Subsequently, two key parameters of A-PCR were optimized through a two-factor cross grouping experiment. The optimized ratio of forward primer to reverse primer and the number of amplification cycles were 150:1 and 36, respectively.

Conclusions

These results contributed to enhancing the efficiency, purity, and yield of ssDNA preparation. Furthermore, statistical analysis identified key factors as primer concentration and ratio, amplification cycles, Mg^{2+} concentration, and annealing temperature. It is recommended that these critical parameters be prioritized during ssDNA preparation by A-PCR in SELEX procedures.

Introduction

Aptamers are novel recognition molecules which can bind to different kinds of targets, including metal ions, small molecular compounds, proteins, viruses, cells, and more [1]. An aptamer is a single-stranded DNA (ssDNA) or RNA molecule selected from an oligonucleotide library using the systematic evolution of ligands by exponential enrichment (SELEX) technique [2]. In addition to exhibiting high affinity and specificity similar to antibodies, aptamers offer several advantages, including the absence of the need for animal or cell experiments, a broader range of target types, simpler and faster synthesis and modification, higher stability, and lower costs [3]. As a result, aptamers have found widespread applications in disease diagnosis and treatment [4, 5], food and environmental detection [6, 7], biological extraction and purification [8, 9], and many other fields [10, 11].

SELEX, the aptamer screening technology, involves repeated rounds of multiple operations [12]. Each round includes several steps: incubation and binding, partitioning and recovery, PCR amplification,

regeneration of single-stranded oligonucleotides, and purification of oligonucleotides [13]. Among these, the preparation of single-stranded oligonucleotides is one of the most critical steps, because producing single stranded oligonucleotides with sufficient purity and quantity in each round significantly influences the successful enrichment of target-bound oligonucleotides in SELEX [14]. Currently, the commonly used methods for preparing single-stranded oligonucleotides in SELEX include thermal denaturation, biotin-streptavidin affinity column separation, denatured gel electrophoresis, exonuclease digestion, and asymmetric PCR (A-PCR) [15]. In addition, various kinds of isothermal amplification techniques, such as rolling circle amplification, strand displacement amplification, and primer exchange reaction, have also been used for large-scale production of ssDNA [16].

A-PCR is a specialized PCR technique designed to produce large quantities of ssDNA instead of double-stranded DNA (dsDNA) [17]. The principle and steps of A-PCR are similar to traditional symmetric PCR, but the main difference lies in the use of a pair of unequal primers, known as the excess primer and the limiting primer. The ratio of these primers is generally between 100:1 and 50:1 [18]. In the presence of template DNA, dNTPs, DNA polymerase, and an appropriate buffer, the amplification reaction proceeds in two stages. In the first stage, dsDNA is generated through exponential amplification with both primers, as the limiting primer is abundant. Once the limiting primer is exhausted, the second stage begins, where the PCR reaction is carried out with only the excess primer, leading to the generation of ssDNA through linear amplification [19]. Apart from the "one-step" method described above, A-PCR can also be performed using a "two-step" method. In the first step, conventional PCR with a pair of equal primers is performed to produce dsDNA. In the second step, this obtained dsDNA serves as the template for PCR with only the excess primer to generate ssDNA [20]. Whether using the "one-step" or "two-step" method, A-PCR combines PCR amplification and ssDNA preparation in a single operation, simplifying the process, reducing costs, and improving efficiency [21]. However, A-PCR is more prone to generating non-specific by-products. Residual dsDNA can interfere with incubation and isolation in subsequent rounds of screening. To address this, optimizing the reaction system and conditions is essential to enhance the yield and purity of ssDNA products [16]. Additionally, exonuclease digestion or gel electrophoresis followed by gel recovery are often employed to further purify the ssDNA and improve its quality [22].

In this study, the PCR amplification procedure was optimized to maximize the yield of ssDNA while minimizing by-products. Initially, single-factor experiments in symmetric PCR were conducted to optimize general conditions such as primer concentration, template concentration, dNTP concentration, Mg^{2+} concentration, DNA polymerase dosage, annealing temperature, and the number of PCR amplification cycles. Following this, a two-factor cross grouping experiment was employed to optimize two critical parameters in A-PCR: the ratio of the two primers and the number of amplification cycles. The optimal A-PCR conditions for ssDNA preparation were established, providing a foundation for the subsequent aptamer screening process. Additionally, the study explored the critical factors affecting A-PCR, which will guide the optimization protocol of A-PCR for more efficient aptamer screening efforts.

Materials and methods

Reagents

All sequences used in this study are listed in Table 1. The ssDNA template was synthesized by Takara Biomedical Technology (Beijing) Co., Ltd. (Beijing, China). It is a ssDNA library consisting of a central randomized region of 40 nucleotides (nt) flanked by two 20-nt primer regions. The forward primer (excess primer) and reverse primer (limiting primer) used in both symmetric PCR and A-PCR were sourced from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The ssDNA library was diluted in 1 x TE buffer to the concentration of 0.4 pmol/uL, while both the forward and reverse primers were also diluted in 1 x TE buffer to the concentration of 5 μM.

Table 1
Sequences used in this study

name	sequences
ssDNA template	5’-AGCAGCACAGAGGTCAGATG-N [a] 40-CCTATGCGTGCTACCGTGAA-3’
forward primer (FP)/ excess primer	5’-AGCAGCACAGAGGTCAGATG-3’
reverse primer (RP)/ limiting primer	5’-P- TTCACGGTAGCACGCATAGG-3’

[a] N represents A, T, C, or G.

The dNTP mixture (each 10 mM), Taq Plus DNA polymerase (5 U/uL), 10 x PCR Buffer, $MgCl_2$ (25 mM), Acryl/Bis 30% Solution (29:1), and urea were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The O'RangeRuler 10 bp DNA Ladder Marker and 6 x Orange DNA Loading Dye were purchased from Thermo Fisher Scientific Inc. (Waltham, USA). The NA-Red nucleic acid fluorescent dye (2000 x) was purchased from Beyotime Biotechnology (Shanghai, China). Other chemicals and reagents of analytical purity were sourced from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Instruments

PCR amplification was carried out using the GeneAmp PCR System 9700 (Applied Biosystems, Inc., Foster City, USA). DNA concentration was measured by NanoDrop-2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). PCR products were detected by polyacrylamide gel electrophoresis using the DYCZ-24DN vertical electrophoresis tank and the DYY-□-2 constant voltage and current electrophoresis apparatus (Beijing Liuyi Biotechnology CO., LTD, Beijing, China). Gel images were captured using the Gel Doc XR□ Imager (BioRad Laboratories, Inc., Hercules, USA).

Symmetric PCR

Symmetric PCR was initially performed to optimize the general conditions and generate dsDNA templates for A-PCR. The symmetric PCR reaction mixture consisted of ssDNA template, dNTP mixture, forward primer, reverse primer, Taq Plus DNA polymerase, 10 x PCR Buffer, $MgCl_2$ solution, and sterile ultrapure water, making up a total volume of 50 μL. The thermal cycling parameters were as follows: pre-denaturation at 95°C for 5 minutes, followed by repeat cycles of denaturation at 95°C for 30 seconds, annealing at approximately 60°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

Single-factor experiments were conducted to optimize the following conditions: final ssDNA template concentration (4.8, 6.4, 8.0, 9.6, 11.2 fmol/μL), final dNTP concentration (20, 40, 60, 80, 100 μM), final concentrations of both forward and reverse primers (20, 40, 60, 80, 100 nM), DNA polymerase dosage (1, 2, 3, 4, 5 U), final Mg^{2+} concentration (0.5, 1, 1.5, 2, 2.5 mM), annealing temperature (61, 62, 63, 64, 65°C), and the number of amplification cycles (12, 14, 16, 18, 20 cycles).

Asymmetric PCR

A-PCR was performed under the optimal conditions and parameters established for symmetric PCR, with the exception of varying the forward-to-reverse primer ratios and the number of PCR amplification cycles. These two conditions were optimized through a two-factor cross grouping experiment. The forward primer concentration was set to the optimal concentration from symmetric PCR, while the reverse primer concentration was gradually diluted to achieve different primer ratios (50:1, 75:1, 100:1, 125:1, 150:1, 175:1, 200:1). The number of PCR amplification cycles (20, 24, 28, 32, 36, 40, 44 cycles) was also optimized as a crucial parameter in A-PCR.

Polyacrylamide gel electrophoresis (PAGE) and image analysis

The dsDNA produced by symmetric PCR was analyzed using 8% native polyacrylamide gel electrophoresis (native PAGE), while the ssDNA generated by A-PCR was examined through 8% denatured polyacrylamide gel electrophoresis containing 7 M urea (denatured PAGE). In the native PAGE gel image, the presence or absence of non-80 bp impurity bands was assessed, and the intensity of the 80 bp dsDNA band was quantified using the relative quantity tool in Image Lab 4.0 software. According to the Image Lab 4.0 user manual, the relative quantity is the ratio of the band volume divided by the reference band volume, which was used to evaluate the results of PCR optimization. The 100 bp band in the 10 bp DNA Ladder Marker was selected as the reference. The relative quantity value of each band was calculated and automatically listed in the “Rel. Quant.” column of the analysis table. In the denatured PAGE gel image, the relative quantity values of the 80 bp dsDNA band and the 80 nt ssDNA band were determined using 1 μL of ssDNA library as a reference. The ratio of the relative quantity value of the 80 nt ssDNA band to that of the 80 bp dsDNA band was defined as the productive rate, which was used to evaluate the results of A-PCR optimization.

Data analysis and graphing

The relative quantity values and productive rates obtained from the optimization experiments under different conditions were statistically analyzed using IBM SPSS Statistics 26. All optimization experiments were conducted in triplicate, and the quantitative results were expressed as mean ± standard deviation. After performing analysis of variance (ANOVA), the data were subjected to Duncan's post hoc multiple comparisons to identify statistically significant differences ($P < 0.05$) [23]. Line charts and column charts were created using OriginPro 8.1.

Results

Optimization of symmetric PCR

Before conducting A-PCR, symmetric PCR optimization was performed to establish the common parameters. First, the impact of different concentrations of PCR components on the generation of dsDNA was assessed. Then, the amplification conditions, such as annealing temperature and the number of amplification cycles, were optimized.

Unlike traditional PCR, the amplification step in SELEX uses an ssDNA-enriched pool as the template instead of dsDNA. Different amounts (0.6, 0.8, 1.0, 1.2, and 1.4 μL) of an ssDNA pool (0.4 pmol/μL) were added to a 50 μL PCR reaction solution, resulting in final ssDNA template concentrations of 4.8, 6.4, 8.0, 9.6, and 11.2 fmol/μL. The 4.8 and 9.6 fmol/μL ssDNA templates exhibited significantly higher Rel. Quant. (relative quantity) values compared to other concentrations. To reduce costs, 4.8 fmol/μL was selected as the optimal final ssDNA template concentration (Fig. 1**(A)** and **Fig. S1**).

(A) Effect of ssDNA template concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 4.8 fmol/μL, Lane 2: 6.4 fmol/μL, Lane 3: 8.0 fmol/μL, Lane 4: 9.6 fmol/μL, Lane 5: 11.2 fmol/μL. (B) Effect of dNTP concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 20 μM, Lane 2: 40 μM, Lane 3: 60 μM, Lane 4: 80 μM, Lane 5: 100 μM. (C) Effect of forward and reverse primers concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 20 nM, Lane 2: 40 nM, Lane 3: 60 nM, Lane 4: 80 nM, Lane 5: 100 nM. (D) Effect of Taq DNA polymerase dosage on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 1 U, Lane 2: 2 U, Lane 3: 3 U, Lane 4: 4 U, Lane 5: 5 U. (E) Effect of Mg^{2+} concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 0.5 mM, Lane 2: 1.0 mM, Lane 3: 1.5 mM, Lane 4: 2.0 mM, Lane 5: 2.5 mM. These line chats show the relative quantity values of dsDNA PCR products with different component concentrations, as estimated by Image Lab 4.0. Error bars represent the standard deviation of 3 replicates. Different lowercase letters (a, b, c) indicate significant differences among different experimental groups ($P < 0.05$). The same letter indicates no significant difference.

Concentrations of 200–250 μM for each dNTP are typically recommended in conventional PCR [24]. Since aptamers, which generally consist of 60–100 nucleotides, are much shorter than biological genes, fewer dNTPs are required for the amplification of the ssDNA aptamer pool. Five different final dNTP concentrations (20, 40, 60, 80, and 100 μM) were tested in symmetric PCR. However, no significant differences were observed in the band intensity across these concentrations. Therefore, the lowest concentration of 20 μM was selected for dNTPs (Fig. 1**(B)** and **Fig. S2**).

One of the most crucial factors influencing the efficiency and specificity of the amplification reaction is the oligonucleotide primers [24]. Optimization was performed using different final concentrations of both primers, ranging from 20 to 100 nM, to achieve high-yield products and suppress the amplification of unwanted sequences. As shown in Fig. 1**(C)** and **Fig. S3**, the generation of 80 bp products increased gradually with the rising concentration of both primers up to 80 nM. Further increasing the primer concentrations to 100 nM did not result in a significant improvement in the generation of the desired products. Therefore, 80 nM was selected as the optimal final primer concentration due to the highest yield of 80 bp products.

Taq polymerase from different manufacturers may vary in terms of yield, product length, and fidelity of amplification [25]. Therefore, it is essential to optimize PCR reactions for each new batch of Taq polymerase. Among the five different enzyme amounts tested (1, 2, 3, 4, and 5 U), a dosage of 2 U of Taq DNA polymerase produced the highest Rel. Quant. value. Since this lower dosage provided the best amplification, 2 U was chosen as the optimal dosage of Taq DNA polymerase for the amplification reaction (Fig. 1**(D)** and **Fig. S4**).

Mg^{2+} is the most effective divalent cation for enhancing thermostable DNA polymerase activity. Since dNTPs and oligonucleotides bind with Mg^{2+}, the molar concentration of Mg^{2+} must exceed the total molar concentration of phosphate groups provided by dNTPs and primers [24]. Therefore, it is crucial to determine the optimal concentration of Mg^{2+} in PCR solutions. As shown in Fig. 1**(E)** and **Fig. S5**, the band intensity increased noticeably within the range of 0.5 to 1.5 mM Mg^{2+}, but no significant increase in band intensity was observed when 2.0 and 2.5 mM Mg^{2+} were used. Thus, the optimal Mg^{2+} concentration was determined to be 1.5 mM.

The temperature used for the annealing step is critical. A higher annealing temperature can lead to a lower yield of amplified products, while a lower annealing temperature may result in the generation of nonspecific products [24]. Among the five different annealing temperatures tested, 62°C was selected as the optimal temperature because it produced a significantly higher band intensity of 80 bp products without obvious by-products (Fig. 2**(A)** and **Fig. S6**).

(A) Effect of annealing temperature on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 61°C, Lane 2: 62°C, Lane 3: 63°C, Lane 4: 64°C, Lane 5: 65°C. (B) Effect of repeat amplification cycle on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 12 cycles, Lane 2: 14 cycles, Lane 3: 16 cycles, Lane 4: 18 cycles, Lane 5: 20 cycles. These line chats show

the relative quantity values of dsDNA PCR products with different conditions, as estimated by Image Lab 4.0. Error bars represent the standard deviation of 3 replicates. Different lowercase letters (a, b, c) indicate significant differences among different experimental groups ($P < 0.05$). The same letter indicates no significant difference.

The required number of amplification cycles depends on the amount of template DNA present at the beginning of the PCR reaction, as well as the efficiency of primer extension and amplification [24]. As shown in Fig. 2**(B)**and **Fig. S7**, the Rel. Quant. value increased rapidly as the number of amplification cycles increased from 12 to 14. However, further increasing the number of cycles to 20 resulted in a decrease in the Rel. Quant. value and an increase in by-products. Therefore, 14 amplification cycles were determined to be optimal for symmetric PCR.

Optimization of A-PCR

The dsDNA generated in symmetric PCR was subsequently used as the template for the A-PCR reactions. Based on the above results of single-factor experiments, the critical conditions, including primer ratios (50:1, 75:1, 100:1, 125:1, 150:1, 175:1, 200:1) and PCR amplification cycles (20, 24, 28, 32, 36, 40, 44 cycles), were selected for two-factor cross grouping experiments to explore their influence on A-RCR. The relative quantity values were obtained using Image Lab 4.0 (**Table S1** and **Fig. S8-S14**), and the productive rates were calculated (**Table S2** and **Fig. S8-S14**).

Firstly, the intensities of the 80 nt ssDNA bands, represented by the relative quantity values, were analyzed by variance. As shown in **Table S3**, the P values for both amplification cycles and primer ratios were close to 0.000, well below the 0.05 threshold, indicating that both amplification cycles and primer ratios had highly significant effects on the band intensity. Furthermore, a significant interaction was observed between amplification cycles and primer ratios, as the P value for the interaction term (cycles * ratios) was also close to 0.000. Duncan's post hoc multiple comparisons revealed that 36 and 40 cycles produced the highest relative quantity values (Fig. 3**(A), Table S5**). Additionally, the primer ratio of 150:1 resulted in noticeably higher band intensity compared to other ratios (Fig. 3**(B), Table S6**).

Next, analysis of variance was performed for the productive rates (**Table S4**). The ANOVA results were consistent with those obtained for the relative quantity values. Duncan's post hoc multiple comparisons also yielded results similar to those of the relative quantity values. Specifically, 36 cycles and the primer ratio of 150:1 generated the highest productive rates, respectively (Fig. 4, **Table S7** and **Table S8**).

Combining the statistical analysis results of relative quantity values and productive rates, it was determined that 36 cycles of amplification and a primer ratio of 150:1 were the optimal conditions for A-PCR.

Verification experiment

To confirm the optimized conditions and parameters, both symmetric PCR and A-PCR were repeated. Symmetric PCR was carried out under the optimized conditions as follows: 4.8 fmol/μL of ssDNA

template, 20 μM of dNTP, 80 nM of forward and reverse primers, 2 U of Taq DNA polymerase, 1.5 mM of Mg^{2+}, 62°C annealing temperature, and 14 amplification cycles. As shown in Fig. 5**(A)**, the Rel. Quant. value of the repeated experiments was 1.038 ± 0.050, with a relative standard deviation (RSD) of 4.833%. A-PCR was performed under the optimal conditions, with a primer ratio of 150:1 and 36 amplification cycles. As shown in Fig. 5**(B)**, the band intensity and productive rate were 0.364 ± 0.025 and 14.075 ± 1.065, respectively, with RSD values of 6.847% and 7.566%. These results demonstrated that the optimized PCR process was stable and reliable.

Discussion

A-PCR is one of the commonly used ssDNA generation methods in the aptamer screening process [26]. In most conventional biological research, PCR uses a single, unique dsDNA template. However, in SELEX, the DNA template is an ssDNA pool, which consists of a vast number of single-stranded sequences with varying GC contents and diverse secondary structures. The high sequence diversity results in significant complexity during the amplification process, such as the formation of smeary bands composed of high molecular weight PCR by-products [27]. Therefore, optimizing PCR conditions is crucial to maximize the production of the required ssDNAs and minimize the generation of undesired by-products.

Herein, through single-factor experiments, the optimal symmetric PCR conditions were determined as follows: the final ssDNA template concentration was 4.8 fmol/μL, the final dNTP concentration was 20 μM, the final concentrations of both primers were 80 nM, the dosage of Taq DNA polymerase was 2 U, the final Mg^{2+} concentration was 1.5 mM, the annealing temperature was 62°C, and the amplification cycle was 14. Through a two-factor cross grouping experiment, the optimal A-PCR conditions were found to be a forward-to-reverse primer ratio of 150:1 and an amplification cycle of 36. The verification test showed that the relative quantity values of the desired dsDNA and ssDNA bands were 1.038 ± 0.050 and 0.364 ± 0.025, respectively. The lower RSD values of 4.833% and 6.847% demonstrated the stability and reliability of the PCR process.

Our long-term experimental investigations demonstrate that primer concentration, primer ratio and amplification cycle number have a significant impact on both the yield and specificity of A-PCR. Statistical analysis of optimized conditions in the current study has confirmed these observations. The results of the optimization experiments revealed that primer concentration/ratio, amplification cycles and Mg^{2+} concentration had the most pronounced impacts on PCR performance, while ssDNA template concentration, Taq DNA polymerase dosage and annealing temperature exhibited more moderate effects. The dNTP concentration, within the range tested in this experiment, did not have a significant impact on PCR.

Conclusion

In this study, two-step asymmetric PCR was optimized to prepare single-strand DNA in aptamer screening. Single-factor experiments were first performed to obtain the optimal general conditions in

symmetric PCR. Then, a two-factor cross grouping experiment was carried out to optimeze two key parameters of A-PCR. Through statistical analysis of the results, the differences in the impact of various experimental conditions on A-PCR were confirmed. These findings provide valuable guidance for future research, demonstrating that optimal A-PCR performance can be achieved through strategic adjustments of these key parameters, thereby eliminating the necessity for additional condition optimizations and significantly reducing experimental workload.

Abbreviations

A-PCR, asymmetric PCR; ANOVA, analysis of variance; denatured PAGE, denatured polyacrylamide gel electrophoresis; dsDNA, double-stranded DNA; native PAGE, native polyacrylamide gel electrophoresis; Rel. Quant., relative quantity; RSD, relative standard deviation; SELEX, systematic evolution of ligands by exponential enrichment; ssDNA, single-stranded DNA.

Declarations

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Competing Interests The authors of this work declare no competing interests.

Author contributions Huajie Gu contributed to the study conception and design, funding acquisition, project administration, resources, supervision. Material preparation, experiments operation, data collection and analysis were performed by Jiaming Shen and Zihan Chen. The original draft of the manuscript was written by Jiaming Shen, Zihan Chen and Huajie Gu. Formal analysis, investigation, manuscript review and editing were carried out by Huajie Gu and Liling Hao. All authors read and approved the final manuscript.

Ethics approval This article contains no research involving human participants and animal subjects.

Supplementary information Supplementary materials, containing all the electrophoretic diagrams and data, the data tables of the two-factor cross grouping experiments and the analysis results of post hoc multiple comparisons using the Duncan method (from IBM SPSS Statistics 26), are available in the online version.

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Figures

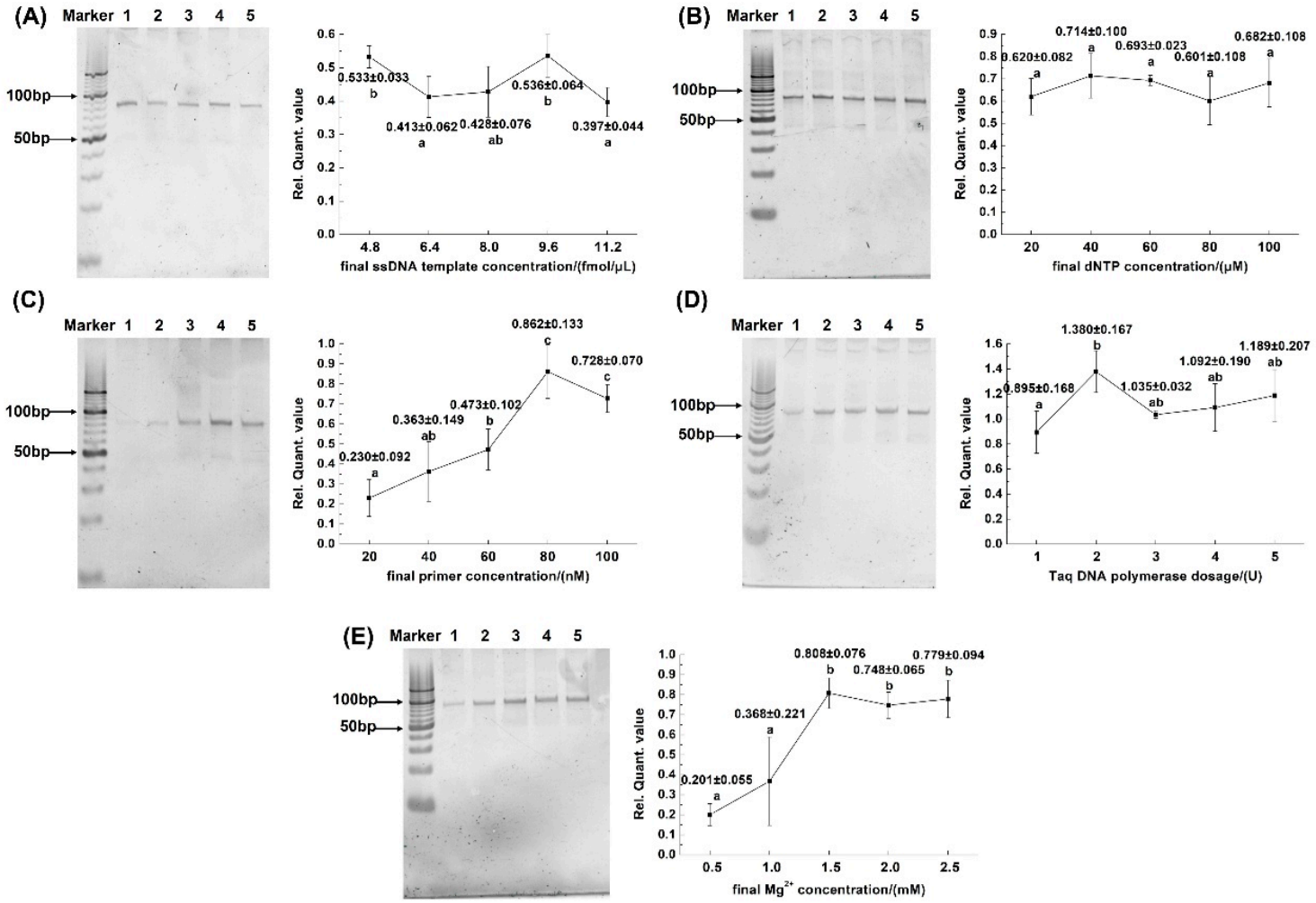


Figure 1

Optimizing the concentrations of different components in symmetric PCR.

(A) Effect of ssDNA template concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 4.8 fmol/μL, Lane 2: 6.4 fmol/μL, Lane 3: 8.0 fmol/μL, Lane 4: 9.6 fmol/μL, Lane 5: 11.2 fmol/μL. (B) Effect of dNTP concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 20 μM, Lane 2: 40 μM, Lane 3: 60 μM, Lane 4: 80 μM, Lane 5: 100 μM. (C) Effect of forward and reverse primers concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 20 nM, Lane 2: 40 nM, Lane 3: 60 nM, Lane 4: 80 nM, Lane 5: 100 nM. (D) Effect of Taq DNA polymerase dosage on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 1 U, Lane 2: 2 U, Lane 3: 3 U, Lane 4: 4 U, Lane 5: 5 U. (E) Effect of Mg^{2+} concentration on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 0.5 mM, Lane 2: 1.0 mM, Lane 3: 1.5 mM, Lane 4: 2.0 mM, Lane 5: 2.5 mM. These line chats show the relative quantity values of dsDNA PCR products with different component concentrations, as estimated by Image Lab 4.0. Error bars represent the standard deviation of 3 replicates. Different lowercase letters (a, b, c) indicate significant differences among different experimental groups ($P<0.05$). The same letter indicates no significant difference.

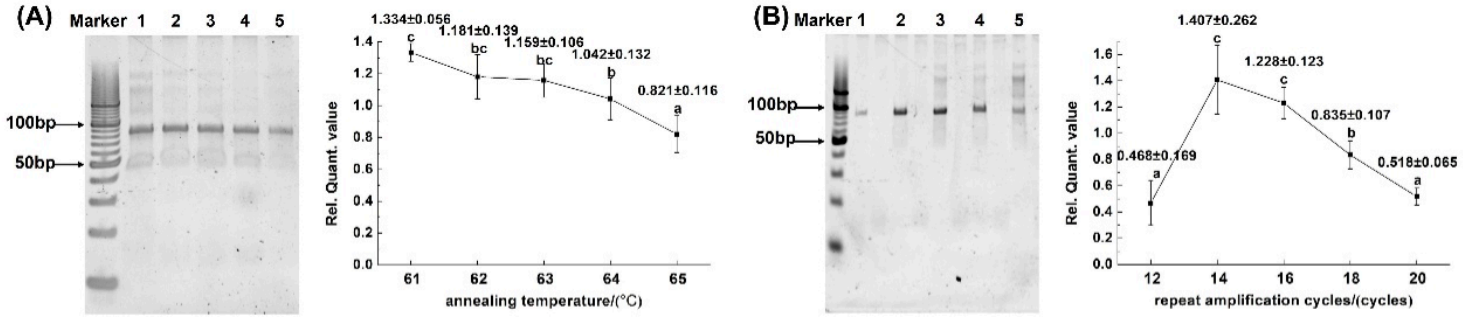


Figure 2

Optimizing the conditions of symmetric PCR.

(A) Effect of annealing temperature on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 61 °C, Lane 2: 62 °C, Lane 3: 63 °C, Lane 4: 64 °C, Lane 5: 65 °C. (B) Effect of repeat amplification cycle on symmetric PCR. In the native PAGE diagram, Marker: 10 bp DNA ladder, Lane 1: 12 cycles, Lane 2: 14 cycles, Lane 3: 16 cycles, Lane 4: 18 cycles, Lane 5: 20 cycles. These line chats show the relative quantity values of dsDNA PCR products with different conditions, as estimated by Image Lab 4.0. Error bars represent the standard deviation of 3 replicates. Different lowercase letters (a, b, c) indicate significant differences among different experimental groups (P<0.05). The same letter indicates no significant difference.

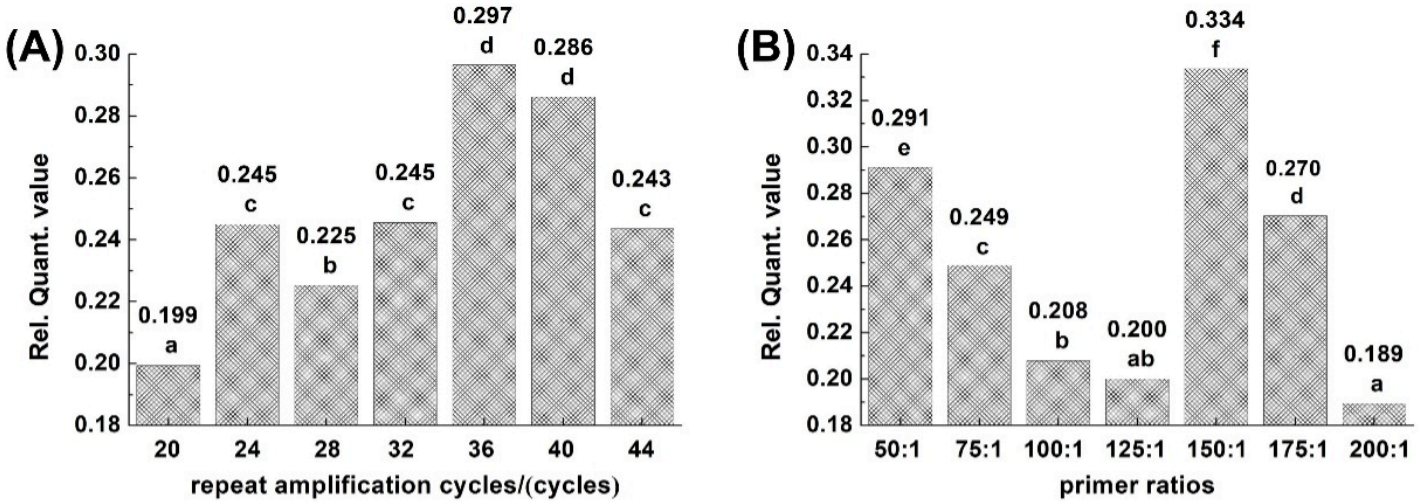


Figure 3

Post hoc multiple comparisons of the relative quantity values among different conditions. (A) among different amplification cycles. (B) among different primer ratios. Different lowercase letters (a, b, c, d, e, f) indicate significant differences among different experimental groups (P<0.05). The same letter indicates no significant difference.

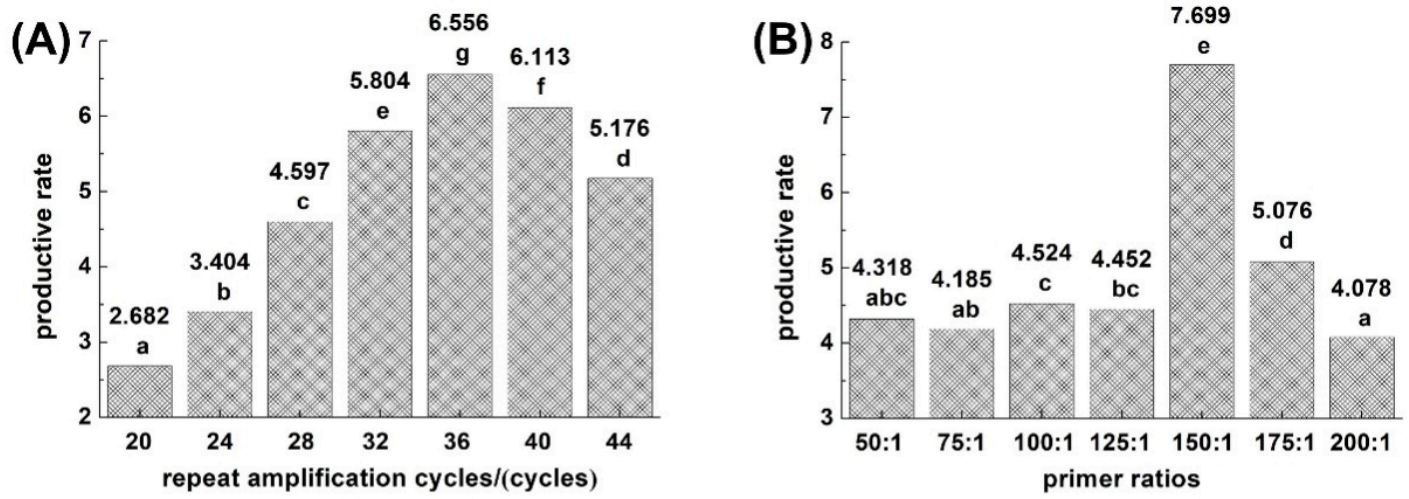


Figure 4

Post hoc multiple comparisons of the productive rates among different conditions. (A) among different amplification cycles. (B) among different primer ratios. Different lowercase letters (a, b, c, d, e, f, g) indicate significant differences among different experimental groups ($P<0.05$). The same letter indicates no significant difference.

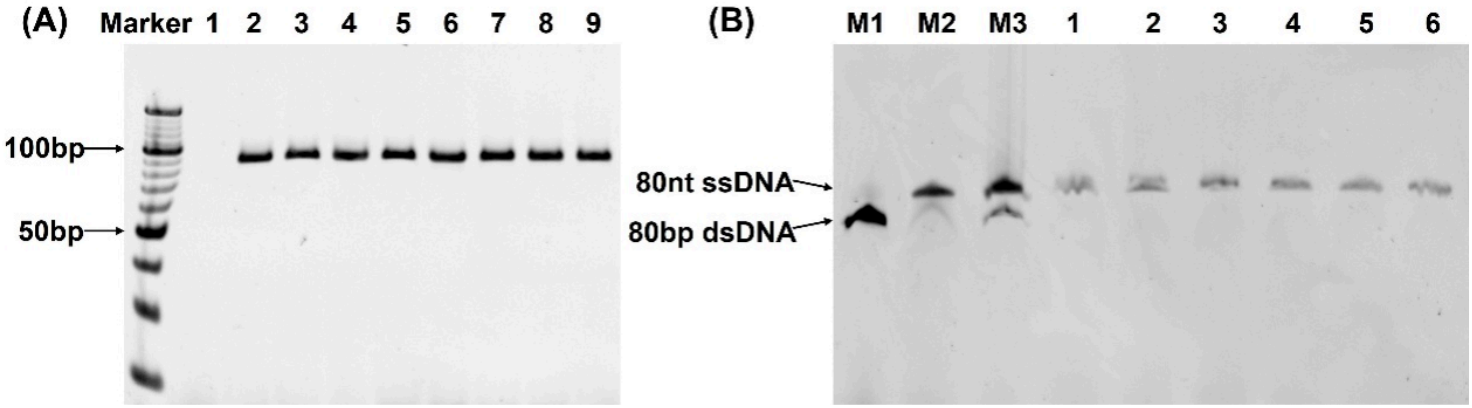


Figure 5

Verification tests of symmetric PCR (A) and A-PCR (B) under the optimized conditions. (A) Native PAGE of symmetric PCR products. Marker: 10 bp DNA ladder, Lane 1: no-template control, Lane 2-9: symmetric PCR under the optimized conditions. (B) Denatured PAGE of A-PCR products. M1: 80 bp dsDNA symmetric PCR products, M2: 80 nt ssDNA library, M3: mixture of 80 bp dsDNA and 80 nt ssDNA, Lane 1-6: A-PCR under the optimized conditions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation20260718.docx