# Construction and optimization of an efficient amplification method of a random ssDNA library by asymmetric emulsion PCR

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## Abstract

Construction of a random ssDNA sublibrary is an important step of the aptamer screening process. The available construction methods include asymmetric PCR, biotin–streptavidin separation, and lambda exonuclease digestions, in which PCR amplification is a key step. The main drawback of PCR amplification is overamplification increasing nonspecific hybridization among different products and by-products, which may cause the loss of potential high-quality aptamers, inefficient screening, and even screening failure. Cycle number optimization in PCR amplification is the main way to avoid overamplification but does not fundamentally eliminate the nonspecific hybridization, and the decreased cycle number may lead to insufficient product amounts. Here, we developed a new method, "asymmetric emulsion PCR," which could overcome the shortcomings of conventional PCR. In asymmetric emulsion PCR, different templates were separated by emulsion particles, allowing single-molecule PCR, in which each template was separately amplified, and the nonspecific hybridization was avoided. Overamplification or formation of by-products was not observed. The method is so simple that direct amplification of 40 or more cycles can provide a high-quality ssDNA library. Therefore, the asymmetric emulsion PCR would improve the screening efficiency of systematic evolution of ligands by exponential enrichment.



***Keywords:*** *asymmetric emulsion PCR, random ssDNA library, SELEX, aptamer*

## 1. Introduction

Aptamers are short ssDNA or RNA molecules that can specifically bind to various target molecules with high affinity, such as proteins, amino acids, drugs, metal ions, and cells. Aptamers have functions similar to those of antibodies, but they can be easily synthesized and conveniently preserved. Therefore, aptamers have an application potential as substitutes of antibodies in clinical diagnosis and disease treatment [1–8]. However, in fundamental research and clinical diagnosis, relevant applications are still dominated by monoclonal antibodies, and aptamer diagnostics is in the experimental stage, because the aptamer screening efficiency of the classical systematic evolution of ligands by exponential enrichment (SELEX) technology is relatively low [9, 10], and high-quality aptamers are not easily available.

Aptamers are screened by the technology of SELEX. In recent years, with the introduction of capillary electrophoresis [11], microfluidic chips [12], emulsion PCR [10], and other technologies, the screening efficiency of aptamers has been improved to some degree. SELEX technology has two main steps: the separation of the bound oligonucleotide sequence and the construction of the ssDNA sublibrary. The construction methods of ssDNA sublibrary mainly include asymmetric PCR

***Abbreviation:*** *SELEX, systematic evolution of ligands by exponential enrichment.*

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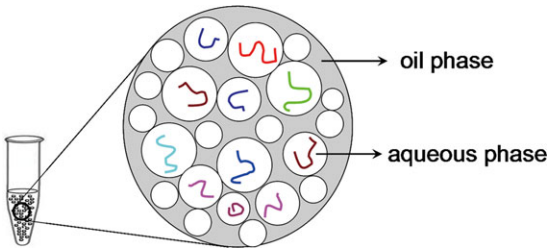


**FIG. 1** *Schematic diagram of asymmetric emulsion PCR. The different types of ssDNA, which marked with different colors, cannot touch each other in the process of PCR amplification. Therefore, hybridization among different kinds of ssDNA does not occur and nonspecific amplification and by-products are avoided.*

[13], biotin–streptavidin separation [14], and lambda exonuclease digestions [15]. In these methods, PCR amplification is a key step. Overamplification and the generation of by-products are common phenomena in PCR amplification [9, 10]. By-products are generated due to the nonspecific hybridization, which may lead to the loss of potential high-quality aptamers and even screening failure. To avoid overamplification, optimization of PCR amplification cycle number is necessary [16]. A decrease in PCR amplification cycle number results in diminished nonspecific hybridization among different products, and the quantity of by-products is reduced. However, this does not fundamentally prevent the nonspecific hybridization among different products, and the decreased cycle number may lead to the formation of an insufficient amount of products.

In this paper, we describe the establishment and optimization of an asymmetric emulsion PCR method. In the asymmetric emulsion PCR technique developed, the different types of ssDNA are separated from each other by emulsion particles and then converted into thousands of small independent amplification tubes to allow the conduction of single-molecule PCR (Fig. 1). In this way, the nonspecific hybridization was avoided, and overamplification or formation of by-products was not observed. A high-quality ssDNA library was obtained after direct amplification of 40 or more cycles.

## 2. Materials and Methods

### 2.1. Reagents and instruments

The reagents in the experiment included Span 80, Tween 80, and mineral oil (Sigma, St. Louis, MO, USA), and Pfu DNA polymerase and dNTP (Sangon, Shanghai, People's Republic of China). An MJ-Mini PCR amplification system (Bio-Rad, CA, USA), a Bio-Rad electrophoresis apparatus (Bio-Rad), and a magnetic stirrer (VWR, West Chester, PA, USA) were used in the experiment.

### 2.2. Experimental methods and procedures

#### *2.2.1. Primers and random ssDNA library*

The random ssDNA library was constructed as 90-mer oligonucleotides, which were composed of two 19-bp fixed sequences on both sides and the 52-bp central random sequence: 5′-GAACATTGGCGTCCGTGAG-$N_{52}$-ACTTCCTCAAACGCCCAA-3′ [10]. The upstream primer was 5′-GAACATTGGCGTCCGTGAG-3′ and the downstream primer was 5′-TTGGGCGTTTGAGGAAGTG-3′. The library was constructed by the Integrated DNA Technologies (Coralville, Johnson, IA, USA) and the primers were synthesized by Shanghai Sangon Biotech (Shanghai, People's Republic of China).

#### *2.2.2. Preparation of emulsion*

The oil phase consisted of 4.5% (v/v) Span 80, 0.4% (v/v) Tween 80, 0.05% (v/v) Triton X-100, and 95.05% (v/v) mineral oil [17]. The aqueous phase was the PCR solution. The emulsion particles (o/w) were prepared according to the following steps. Within 1 Min, 0.1 mL of the PCR solution was uniformly dropped into a 2-mL flat frozen pipe containing 0.2 mL of the oil phase (8–10 μL per drop). Meanwhile, the emulsion was stirred with a magnetic stirrer (8 × 1.5 $mm^2$, 1,500 rpm). After the PCR solution was added into the pipe, the emulsion was stirred for 20 Min for immediate PCR amplification.

#### *2.2.3. Asymmetric PCR*

The 50 μL asymmetric PCR system contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.6), 2.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTP, 0.4 μmol/L upstream primer, 0.02 μmol/L downstream primer, 0.04 μg/L template, and 100 U/μL Pfu DNA polymerase. The asymmetric PCR procedure was composed of 30-Sec denaturation at 94 °C, 35 cycles of 40-Sec denaturation at 94 °C, 30-Sec annealing at 60 °C, 30-Sec extension at 72 °C, and 3-Min final extension at 72 °C. The number of cycles to be optimized ranged from 10 to 50.

#### *2.2.4. Asymmetric emulsion PCR*

The ssDNA library was used as a template. The 100 μL emulsion aliquot utilized in the asymmetric PCR consisted of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.6), 2.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTP, 0.4 μmol/L upstream primer, 0.02 μmol/L downstream primer, 0.004 μg/L template, and 100 U/μL Pfu DNA polymerase. In the optimization experiments, six template concentrations were set as follows: 0.0004, 0.004, 0.04, 0.4, 4, and 40 μg/mL; five upstream primer concentrations were employed: 0.2, 0.4, 0.6, 0.8, and 1. Four downstream primer concentrations were utilized as follows: 0.02, 0.01, 0.005, 0.0025. The asymmetric emulsion PCR was performed as follows: 30-Sec denaturation at 94 °C, 35 cycles of 40-Sec denaturation at 94 °C, 30-Sec annealing at 60 °C, 30-Sec extension at 72 °C, and 3-Min final extension at 72 °C. The cycle number to be optimized ranged from 10 to 50.

#### *2.2.5. Recovery of PCR products from the emulsion*

After the PCR solution was centrifuged at 9,000*g* for 6 Min, the upper oil phase was removed. Then, water-saturated ether with the same volume was added into the PCR solution. The obtained PCR solution was vortexed and then centrifuged at 9,000*g* for 20 Sec. After removing the upper ether phase, the lids of frozen pipes were opened at room temperature.

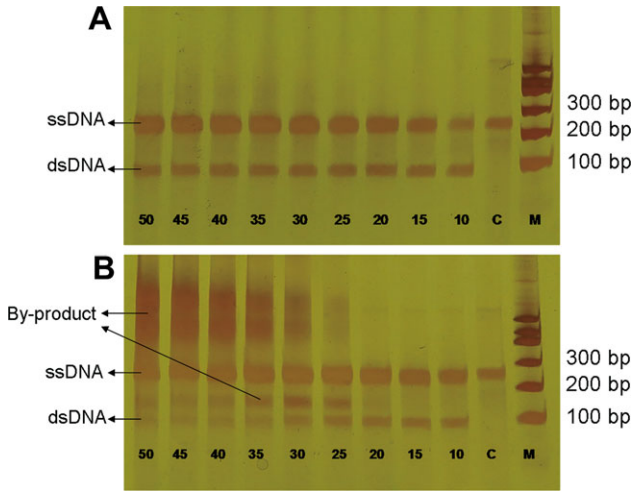


**FIG. 2** *(A) The ssDNA library obtained by asymmetric emulsion PCR. The PCR amplification cycle number ranged from 10 to 50, and the control was the ssDNA library. When the cycle number was increased from 10 to 50, sufficient amounts of products were generated and no by-product was produced. (B) The ssDNA library obtained by conventional asymmetric PCR. The PCR amplification cycle number ranged from 10 to 50, and the control was the ssDNA library. By-products appeared after 20 cycles. A large amount of by-products were generated after 25 cycles, and they could not be separated from products.*

Next, to evaporate residual ether, the opened pipes were left undisturbed at room temperature for 10 Min.

### *2.2.6. Analysis of PCR products*

PCR products were analyzed by denaturing PAGE and silver staining.

## 3. Results

### 3.1. Comparison between conventional asymmetric PCR and asymmetric emulsion PCR

In asymmetric emulsion PCR, after 50 cycles, no by-product was formed, and the amounts of target products were significantly increased (Fig. 2A). Conventional asymmetric PCR produced sufficient ssDNA sublibrary, but by-products were generated after 20 cycles. After 25 cycles, the bands of target products were mixed with the bands of by-products, and the product purification was extremely difficult (Fig. 2B).

### 3.2. Optimization of asymmetric emulsion PCR

#### *3.2.1. Optimization of the template concentration*

The augmentation of template concentration from 0.0004 to 0.04 μg/mL caused a rise in the yield of products, whereas a template concentration above 0.04 μg/mL did not result in greater product formation. At a template concentration of 4 μg/mL, a small amount of by-products was generated (Fig. 3), whereas at the range 0.04–0.4 μg/mL, the generation of sufficient product quantities was induced, and no by-product was

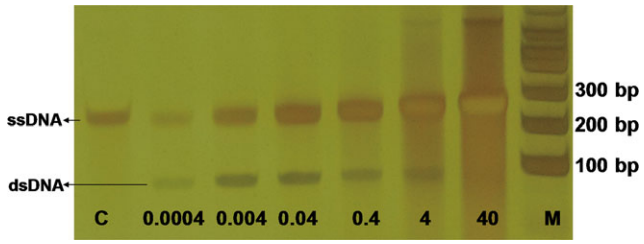


**FIG. 3** *Optimization of template concentration in asymmetric emulsion PCR. The template concentration ranged from 0.0004 to 40 μg/mL, and the control was the ssDNA library. When the template concentration was increased from 0.0004 to 0.04 μg/mL, the amount of products was higher. When the template concentration was elevated above 0.04 μg/mL, the amount of products showed no significant change. The template concentration of 4 μg/mL induced the generation of a large amount of by-products. In the template concentration range of 0.04–0.4 μg/mL, sufficient quantities of products were yielded and no by-product was produced.*

produced. Therefore, the applicable template concentration range was 0.04–0.4 μg/mL.

#### *3.2.2. Optimization of the primer concentration*

ssDNA is an asymmetric PCR product. When the concentration of primers is too high, they tend to react with ssDNA and form nonspecific amplification products. Therefore, it is necessary to optimize both the ratio between the upstream primer and the downstream primer and their absolute concentrations. As shown in Fig. 4A, when the concentration of the upstream primer was 0.6 μmol/L, a small amount of by-products was generated, but when it reached 1 μmol/L, a large amount of by-products was formed. When the concentration of upstream primer ranged from 0.3 to 0.4 μmol/L, sufficient quantities of products were yielded and no by-product was produced. During the optimization of downstream primer concentration, the upstream primer concentration was 0.4 μmol/L; four downstream primer concentrations were set as follows: 0.02, 0.01, 0.005, and 0.0025 μmol/L. As illustrated in Fig. 4B, under the four downstream primer concentrations, no by-product was generated, but the amount of products was increased with the elevation in the downstream primer concentration. Therefore, 0.02 μmol/L was selected as the optimal downstream primer concentration.

## 4. Discussion

Construction of a random ssDNA sublibrary is important in the entire process of aptamer screening. The successful screening of aptamers is mainly determined by the availability of a high-quality random ssDNA sublibrary. A random ssDNA sublibrary is usually constructed in two steps. First, a dsDNA library was amplified by PCR. Then, the dsDNA library was converted into an ssDNA library [16]. During the PCR amplification,

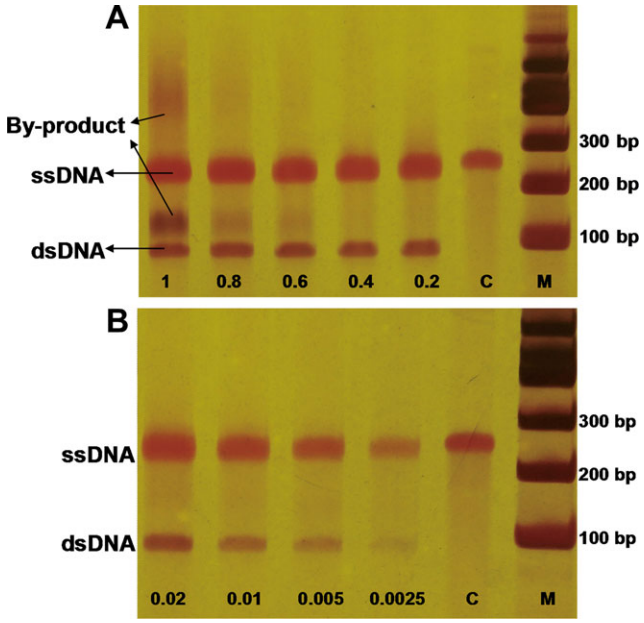


**FIG. 4** *(A) Optimization of the upstream primer concentration. The upstream primer concentration ranged from 0.2 to 1 μmol/L, and the downstream concentration was 0.02 μmol/L. The control was the ssDNA library. When the upstream primer concentration was 0.6 μmol/L, a small amount of by-products appeared. At an upstream primer concentration of 1 μmol/L, a large amount of by-products appeared. In the upstream primer concentration range of 0.2–0.4 μmol/L, sufficient products were generated and no by-product was produced. (B) Optimization of the downstream primer concentration. The downstream primer concentration ranged from 0.0025 to 0.02 μmol/L, and the upstream concentration was 0.4 μmol/L. The control was the ssDNA library. The amount of products was increased with the augmentation in the upstream concentration and no by-product was produced.*

the product yield was low, and the formation of by-products could not be avoided [10]. Musheev and Krylov [9] reduced the quantity of by-products by lowering the number of cycles and optimizing the enzyme concentration. Kang et al. [18] added dimethyl sulfoxide (DMSO) and betaine into the PCR reaction system to reduce the amounts of by-products. Jie and co-workers [19] obtained pure products through separating them from by-products by using capillary electrophoresis. He et al. [20] directly developed an ssDNA library through optimizing the number of cycles of a single-primer PCR system. However, these methods could not fundamentally solve the problem of by-products generation. The overamplification principle is interpreted as follows: The large number of amplification cycles leads to the excessive formation of products and an increase in the probability of nonspecific hybridization among different products, which lead to yielding of by-products and a decrease in products. However, the decrease in the number of cycles could only reduce nonspecific hybridization among different products, and the problem with by-products could not be fundamentally solved. In the asymmetric emulsion PCR system established in our investigation and described in detail in the paper, different templates were separated by emulsion particles to allow single-molecule PCR, in which every template was separately amplified. Asymmetric emulsion PCR cannot only avoid the by-products, but also exclude the separation step of the ssDNA from dsDNA. Asymmetric emulsion PCR directly produces a large amount of ssDNA. In this way, the synthesis procedure of the ssDNA was simplified.

Moreover, we compared conventional asymmetric PCR and asymmetric emulsion PCR. After 25 cycles of conventional asymmetric PCR, a large number of by-products were generated, and the product purification was extremely difficult. In the asymmetric emulsion PCR, after 50 cycles, no by-product was formed. Therefore, the asymmetric emulsion PCR method effectively avoided the generation of by-products and improved the quality and quantity of the ssDNA sublibrary.

During the optimization of asymmetric emulsion PCR, we initially optimized the template concentration. Under the ideal conditions, an emulsion particle contained only one template. When the number of templates present in an emulsion particle was increased, the outcome of the asymmetric emulsion PCR was closer to that of the conventional asymmetric PCR. The increase in template concentration from 0.0004 to 0.04 μg/mL resulted in a higher amount of products, but its elevation above 0.04 μg/mL did not exert this effect. At a template concentration of 4 μg/mL, a small amount of by-products was generated, indicating that when the amount of template was significantly higher than the number of emulsion particles, an emulsion particle contained more templates, leading to the generation of by-products.

During the optimization of the primer concentration, we optimized both the ratio of the upstream primer to the downstream primer and their concentrations. The primers were prone to react with ssDNA, and their higher concentration enhanced the formation of nonspecific hybridization products, as described earlier [10]. Therefore, we first optimized the concentration of upstream primer. When the upstream primer concentration varied from 0.2 to 1 μmol/L, the amount of products showed no significant change; when the upstream concentration of primers was 0.6 μmol/L, a small quantity of by-products was generated, whereas when it was 1 μmol/L, a large amount of by-products was generated. During the optimization of downstream primers, upstream primers concentration was 0.4 μmol/L, and downstream primer concentrations ranged from 0.0025 to 0.02 μmol/L. When the concentration of downstream primers increased, the amount of products increased gradually and no by-product was generated. The downstream primer concentration directly determined the amount of products, and the higher upstream primer concentration led to the generation of by-products.

In short, in the established asymmetric emulsion PCR method, the problem of overamplification and formation of

by-products existing in conventional PCR was solved. The method described here could be used to easily, conveniently, and efficiently construct an ssDNA sublibrary, thus improving the screening efficiency of aptamers and promoting the development of the SELEX technology.

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