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. © 2015 The Authors. Biotechnology and Applied Biochemistry published by Wiley Periodicals, Inc. on behalf of the International Union of Biochemistry and Molecular Biology, Inc. Volume 64, Number 2, Pages 239–243, 2017

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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
Asymmetric Amulsion PCR Random ssDNA Library

mer oligonucleotides, which were composed of two 19-bp fixed sequences on both sides and the 52-bp central random sequence: 5'-GAACATTGGCGTCCGAG-N52-ACTTCTCTAAACGCCC-3' [10]. The upstream primer was 5'-GAACATTGGCGTCCGAG-3' and the downstream primer was 5'-TGGGCGGTAGAAGGTCG-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 mm², 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 MgCl2, 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 MgCl2, 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 9,000g 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 9,000g for 20 Sec. After removing the upper ether phase, the lids of frozen pipes were opened at room temperature.

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.

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-
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 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,
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 µmol/L 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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6. References
[1] Ma, H., Liu, J., Ali, M. M., Mahmood, M. A., Labanieh, L., Lu, M., Iqbal, S. M., Zhang, Q., Zhao, W., and Wan, Y. (2015) Chem. Soc. Rev. 44, 1240–1256.
[2] Sun, H., and Zu, Y. (2015) Small 11, 2352–2364.
[3] Toh, S. Y., Citartan, M., Gopinath, S. C., and Tang, T. H. (2015) Biosens. Bioelectron. 64, 392–403.
[4] Kim, K., Lee, S., Ryu, S., and Han, D. (2014) Biochem. Biophys. Res. Commun. 448, 114–119.
[5] Wu, X., Chen, J., Wu, M., and Zhao, J. X. (2015) Theranostics 5, 322–344.
[6] Xi, Z., Huang, R., Li, Z., He, N., Wang, T., Su, E., and Deng, Y. (2015) ACS Appl. Mater. Interfaces 7, 11216–11223.
[7] Ozalp, V. C., Kavruk, M., Dilek, O., and Bayrac, A. T. (2015) Curr. Top. Med. Chem. 15, 1125–1137.
[8] Lau, I. P., Ngan, E. K., Loo, J. F., Suen, Y. K., Ho, H. P., and Kong, S. K. (2010) Biochem. Biophys. Res. Commun. 395, 560–564.
[9] Musheev, M. U., and Krylov, S. N. (2006) Anal. Chim. Acta 564, 91–96.
[10] Shao, K., Ding, W., Wang, F., Li, H., Ma, D., and Wang, H. (2011) PLoS One 6, e24910.
[11] Drabovich, A., Berezovski, M., and Krylov, S. N. (2005) J. Am. Chem. Soc. 127, 11224–11225.
[12] Lin, H., Zhang, W., Jia, S., Guan, Z., Yang, C. J., and Zhu, Z. (2014) Biomicrofluidics 8, 041501.
[13] Yunusov, D., So, M., Shayan, S., Okhonin, V., Musheev, M. U., Berezovski, M. V., and Krylov, S. N. (2009) Anal. Chim. Acta 631, 102–107.
[14] Stevenson, R., Baxter, H. C., Allken, A., Brown, T., and Baxter, R. L. (2008) Bioorg. Chem. 36, 215–219.
[15] Dastjerdi, K., Tabar, G. H., Dehghani, H., and Haghiparast, A. (2011) Biotechnol. Appl. Biochem. 58, 226–230.
[16] Marimuthu, C., Tang, T. H., Tominaga, J., Tan, S. C., and Gopinath, S. C. (2012) Analyst 137, 1307–1315.
[17] Tawfik, D. S., and Griffiths, A. D. (1998) Nat. Biotechnol. 16, 652–656.
[18] Kang, J., Lee, M. S., and Gorenstein, D. G. (2005) J. Biochem. Biophys. Methods 64, 147–151.
[19] Ying, J., Qing-Qing, W., Jie, F., Xin, G., and Hai-Feng, S. (2010) Chinese J. Anal. Chem. 38, 622–626.
[20] He, C. Z., Zhang, K. H., Wang, T., Wan, Q. S., Hu, P. P., Hu, M. D., Huang, D. Q., Lv, N. H. (2013) Anal. Biochem. 440, 63–70.