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Full Length Research Paper

A simplified system without purification for selection of aptamers against *Vibrio alginolyticus*

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We have explored a simplified system without purification for systematic evolution of ligands by exponential enrichment (SELEX) against *Vibrio alginolyticus* to select single-stranded DNA ligands (aptamers) from a random 82-nt library. The DNA content was quantified by agarose gel electrophoresis and follow-up image analyses with the BandScan 5.0 software. Anti-digoxigenin/HRP system was used to determine the binding activity of aptamers for *V. alginolyticus.* The results showed that the affinity of aptamers increased gradually with the increase of screening rounds, indicating that the lack of purification did not affect screening results but rather made SELEX screening much more convenient and significantly increased the efficiency.

Key words: Aptamers, purification, systematic evolution of ligands by exponential enrichment (SELEX), *Vibrio alginolyticus*.

INTRODUCTION

Systematic evolution of ligands by exponential enrichment (SELEX) is a new combinatorial chemistry technology established in the early 1990s (Robertson and Joyce, 1990; Tuerk and Cold, 1990). A random oligonucleotide library is synthesized and incubated with the target. The sequences which meet the selection standard are recovered and then amplified by polymerase chain reaction (PCR) while the others are discarded. A further enriched pool of selected oligonucleotides is generated by preparation of the relevant single-stranded DNA from the PCR products. These steps are repeated for several times and finally the sequences which best fit the selection criteria are preferentially enriched (Figure 1). The DNA sequences which can bind to a specific target molecule are called aptamers. These short single-stranded nucleic acid oligomers can fold into a vast array of different

three-dimensional structures including hairpins, pseudoknots, bulges, stems, loops, triplexes, and G-quadruplexes (Clark and Remcho, 2002). Because of the variety of their three-dimensional structures, they can interact with almost all types of targets in nature to form a stable compound through hydrogen bond, van der Waals force or electrostatic action (Brockstedt et al., 2004). Based on the above principle, the SELEX technique has been quickly applied in all areas of life sciences and generated a large number of aptamers for clinical research and diagnosis (Shtatland et al., 2000).

Aptamers and antibodies recognize the target molecule in a similar way and they can both be used as detection molecules, but when compared with antibodies, aptamers have the following advantages: higher specificity, a wider range of target molecules, no immunogenicity and no requirement for the target to have immunogenicity either, and smaller molecular weight which help them to quickly enter the organism (Jayasena, 1999; Bruno, 1997). So far aptamers have been successfully generated against many different targets, including small molecules, peptides, monomeric proteins, cofactors, nucleic acids, and viruses, and applied to relevant target identification (Stoltenburg et al., 2007).

In the process of screening aptamers, the tradition way is to use the recovered and purified PCR products as the oligonucleotide pool for the next round. Because the oligonucleotides of SELEX library are usually short (<200-nt), the purification efficiency of PCR products is relatively low and result in loss of some aptamers, especially

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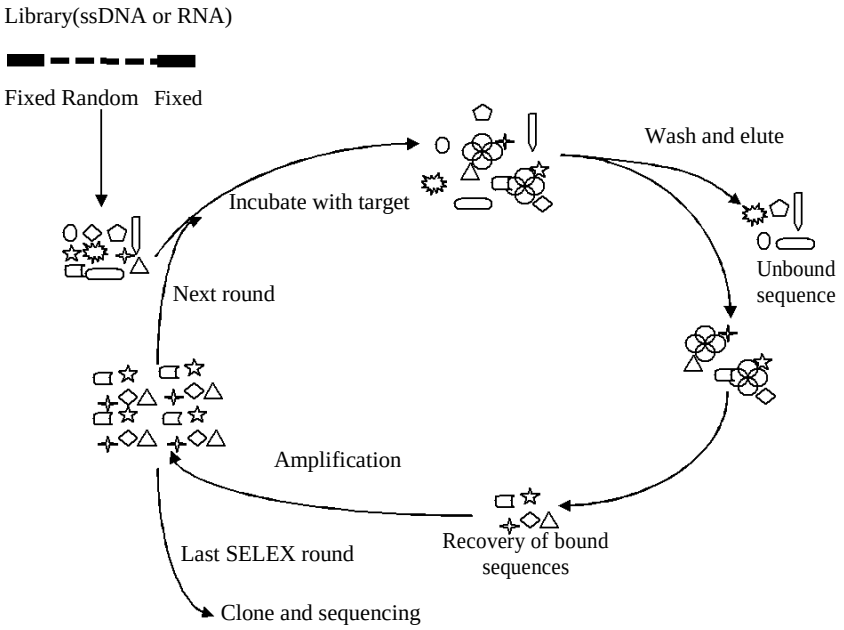

Figure 1. General principle of SELEX (Pestourie et al., 2000)

those with high affinities, which significantly affect the screening results. In addition, the purification is expensive, time-consuming and labor-intensive, all of which limit the application of SELEX.

This study is based on our previous report (Zheng et al., 2010) and explores a simplified system without purification for the selection of aptamers against *Vibrio alginolyticus*, an important aquaculture pathogenic bacterium (Liu et al., 2004; Lee et al., 1996; Lee 1995). This method not only avoids losing aptamers with high affinity, but also simplifies the tedious purification and greatly improves the screening efficiency.

MATERIALS AND METHODS

V. alginolyticus was provided by pathogenic biological laboratory of our school; Taq DNA polymerase, 10× buffer, $MgCl_2$ were purchased from Fermentas; dNTP was purchased from Generay Biotech Co.; Rabbit Anti-digoxin IgG/HRP was purchased from Beijing Biosynthesis Biotech Co.; 3,3',5,5' tetramethylbenzidine (TMB) was purchased from Amresco Co.; 50 bp DNA Ladder was purchased from TianGen Biotech Co.; agarose was purchased from Xiamen Sunma Biotech Co.

Construction of a random ssDNA library

The library of synthetic DNA oligodeoxyribonucleotides with the sequence of 5'-TCA GTC GCT TCG CCG TCT CCT TC—N_{35}—GCA CAA GAG GGA GAC CCC AGA GGG-3' was 82 bases long and contained 35 random nucleotides in the center, which were flanked by two constant regions used for PCR amplification and cloning. The forward primers used for PCR was P1 (5'- TCA GTC GCT TCG CCG TCT CCT TC-3') and the reverse primer was P2 (5'- CCC TCT GGG GTC TCC CTC TTG TGC-3'). Digoxigenin 5'-end modified forward primer P3 (5'-Digoxigenin-TCA GTC GCT TCG CCG TCT CCT TC-3') was used to label the aptamer library. They were all synthesized by Sangon Biotech.

Selection protocol

In the first round of SELEX, 30 µl of ssDNA (10 µM) were diluted with 2× binding buffer (100 mmol/L NaCl, 5 mM KCl, 50 mM Tris-HCl, 1 mM $MgCl_2$, pH7.4) to 3 µM. After denaturalized at 95°C for 5 min and ice bath for 10 min, 500 µl of *V. alginolyticus* (8×10^8) was added and incubated for 30 min at 30°C on a rotary shaker (100 rpm) to precipitate formed complexes. The tube was centrifuged at 6000 rpm for 5 min for the collection of the precipitate, and the supernatant containing free ssDNA was discarded. The precipitate was washed three times with 1 × binding buffer to remove unattached ssDNA, then was added 100 µl 1× binding buffer and heated at 95°C for 5 min followed by centrifugation at 15000 rpm for 10 min. The supernatant was then collected as the template for an asymmetric PCR. After the PCR products were analyzed by agarose gel electrophresis, 100 µl of PCR products were used for another round of SELEX without purification.

Affinity analyses

Firstly the affinity of PCR products was analysed. PCR products of the 3rd, 9^{th} and 15th rounds of SELEX (50 µl each) and a blank control without any PCR product was selected. After denaturalized at 95°C for 5 min and ice bath for 10 min, 500 µl of *V. alginolyticus* (8×10^8) was added and left for 30 min on a rotary shaker to precipitate formed complexes. The tube was centrifuged at 6000 rpm for 5 min for the collection of the precipitate, and the supernatant containing free ssDNA was discarded. The precipitate was washed once with 1 × binding buffer and then Rabbit Anti-digoxin IgG/HRP (1:1000) was added to the tube. After 10 min reaction, the tube was centrifugated and the supernatant was discarded. Then the tube was washed three times with 1 × binding

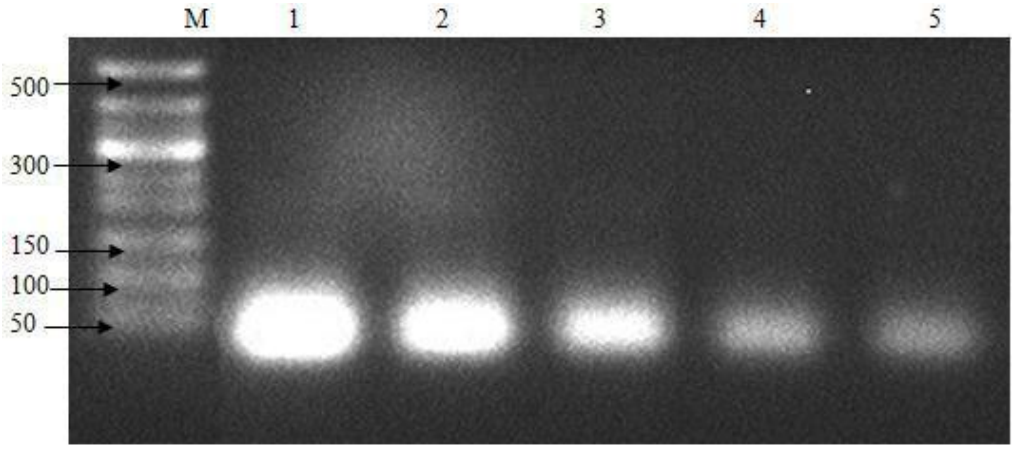

Figure 2. Electrophoresis image of different concentrations of standard ssDNAs.

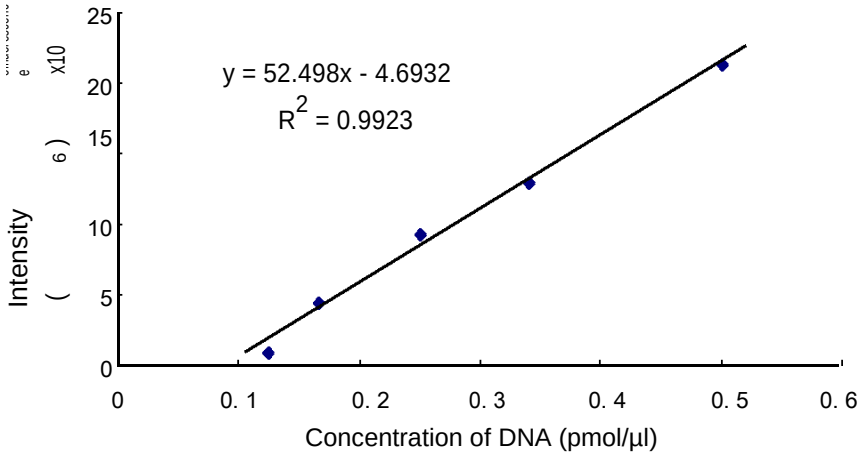

Figure 3. The linear relationship between standard DNA concentrations and luminance values of electrophoresis bands.

buffer. The freshly prepared 200 µl substrate (1 mg/ml TMB: substrate buffer: 30% H_2O_2 = 100:900:1) for IgG/HRP was added to the tube and incubated for 10 min in dark, then 200 µl 2 mol/l H_2SO_4 was added to stop the reaction. The color reaction then was read at 450 nm using a CliniBio Elisa Reader (Bruno and Kiel, 1999). The affinity of the corresponding PCR product is OD_{450} of the study group minus OD_{450} of the control group.

Then the DNA contents in the PCR products was quantified. EB fluorescence spectrophotometry was used to quantify DNA contents of the PCR products as previously described (Niu, 2005). Briefly stated, first, 5 µl standard DNA of different concentrations underwent agarose gel electrophoresis (2.5%) and the ultraviolet images were analyzed by BandScan 5.0 software to obtain the linear relationship between standard DNA concentrations and luminance values of electrophoresis bands. Second, PCR products of the 3rd, 9th and 15th rounds of SELEX and known concentrations of standard DNA underwent agarose gels electrophoresis as described above and the images were similarly analyzed by BandScan 5.0 software. The OD values of the PCR products were converted to the concentration and moles of their DNA contents by the established linear relationship. Finally, the affinity of unit DNA was calculated as follows, Affinity (OD_{450}/nmol) = ($OD_{study\ group}$ - $OD_{control\ group}$)/DNA mole.

Statistical analysis

Data were calculated and analyzed by mean, standard and t-test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Relationship between DNA concentrations and luminance values of electrophoresis bands

As shown in Figure 2, the concentration of DNA decreased gradually as the luminance of electrophoresis bands weakened. The analyses by BandScan 5.0 software showed that the luminance values of electrophoresis bands were in good linear relationships with the concentration of DNA (Figure 3). The standard

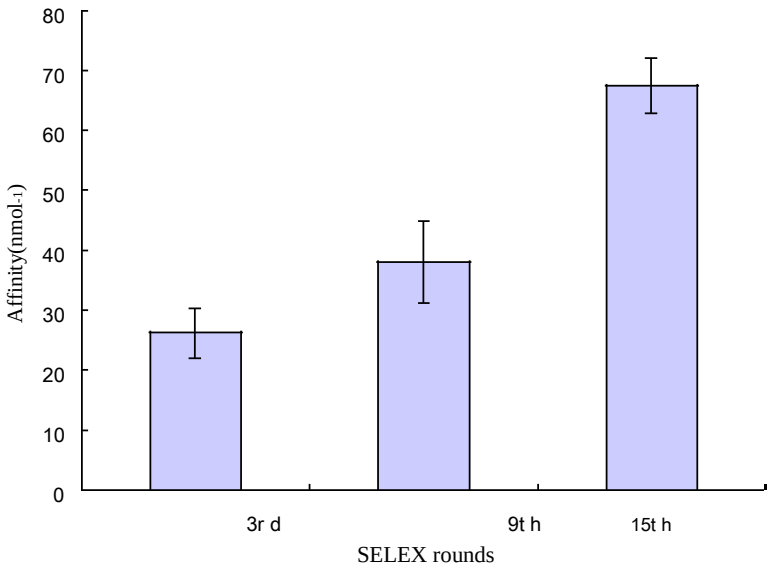

Figure 4. The affinity of aptamers improves as SELEX continues.

curve equation was Y = 52.498X - 4.6932 (Y: luminance values obtained from BandScan 5.0 software/10^6; X: concentration of DNA/pmol·μl^{-1}) with correlation coefficient R^2 of 0.9923. Based on this equation, the DNA concentration of PCR products of the 3rd, 9th and 15th rounds of SELEX were 0.161, 0.148, 0.188 pmol/μl, respectively. The corresponding number of moles were 8.07, 7.42 and 9.42 pmol.

Affinity changes with the SELEX rounds

As shown in Figure 4, as the SELEX screening continued without purification, the affinity of the PCR products for *V. alginolyticus* increased significantly ($p<0.05$) from 26.23 to 67.50 nmol^{-1}. The result indicated that the lack of purification did not affect screening but rather made SELEX screening much more convenient and significantly increased the efficiency.

DISCUSSION

In the commonly used SELEX screening, PCR products were purified for the next round. Because the library are usually short (less than 200-nt), the purification and recovery efficiency of PCR products is relatively low and result in loss of some aptamers, especially those with high affinities, which significantly affect the follow-up screening results. Therefore, in this study we aimed to improve SELEX screening by directly using the amplified PCR product as the library for next screening without purification.

This improvement is mainly based on the following considerations. In PCR products, ssDNA are the majority, so the PCR products screening combined with bacteria can be actually considered as ssDNA screening combined with bacteria. If some ssDNA combine with some impurities like primers and polymerases, the subsequent centrifugation can remove these ssDNA. If certain impurities compete with ssDNA to combine with bacteria, as the screening continues, those ssDNA with low affinity will be gradually discarded and the aptamers with high affinity will be kept. Therefore, even if some substances in the PCR products indeed interfere with the screening, they will not affect the final results but instead can improve the screening efficiency.

For affinity assays, we used the affinity of unit DNA mole to evaluate the affinity of oligonucleotide library, which avoids the issue of low proportion of aptamers in the total PCR products. In the current study we adopted EB fluorescence spectrophotmetry to determine concentration of DNA of the PCR products. This method is quick, direct, and easy to operate. It also requires only small amounts of sample and can analyze DNA of PCR products by gel electrophoresis. The use of BandScan software to analyze electrophoresis data can avoid man-made errors and reduce the total experimental error, showing good accuracy and reliability.

In summary, using PCR products instead of purified oligonucleotide library for SELEX helps simplify the operation, raise the efficiency, and avoid unexpected loss of aptamers, all of which significantly improves the stability and reliability of the procedure.

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