Method of mRNA Display for Selecting Peptides that Bind with Target Enzyme RNA

Yan Song [1, 2], Wang Zheng [1], Yang Shao-li [3], Zhang Yi [2] & Lin Xiu-kun [1]

[1]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China ; [2] School of Environmental Science and Engineering，Dalian Jiaotong University，Dalian 116028，China; Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences, Yantai, 264003, China

Correspondence should be addressed to Yan Song (yansong209@yahoo.com.cn) and Lin Xiu-kun (linxiukun@yahoo.com).

Abstract— **In *vitro* display techniques are useful tools for selecting novel ligands that bind to target biomolecules. In this paper, mRNA display method was used to select peptides that bind thymidylate synthase RNA with high affinity from a peptide library. We provide a detailed analysis of the critical steps of an mRNA-display-based selection, including the generation of an mRNA-displayed library, formation of RNA-peptide fusions, and selection of RNA affinity peptides. After optimization of the experimental steps, the efficiency of RNA-linker purification and the reverse transcription was improved, and the unspecified binding peptides were removed. Our study could facilitate the use of mRNA display in the field of biotechnology, medicine and proteomics.**

Keywords- in vitro mRNA display ; selection ; affinity peptide

1. Introduction

In recent years, display technology is becoming one of the most widely used tools for the selection of functional proteins or peptides, and now being applied to the field of drug discovery. *In vitro* display systems are not limited by cellular transformation efficiencies; thus, very large libraries of up to 10^{13}-10^{14} members can be built. In mRNA display, a library of genotype–phenotype linking molecules is constructed in which mRNA binds to protein through puromycin during cell-free translation. After affinity selection, the selected molecules can be amplified by means of RT–PCR. By performing iterative selection, very low copy number proteins can be detected from large-scale cDNA libraries. As an *in vitro* selection approach, mRNA display technique has identified many functional peptides, such as TNF-α (tumor necrosis factor alpha) [1] and DNA-binding protein [2].We anticipate that mRNA display will have a great impact on applications in biotechnology, medicine and proteomics.

2. Materials and methods

2.1 PCR amplification

(i) dsDNA library was a gift from Howard Hughes Medical Institute, USA. The oligonucleotide sequences were as follows: TTC TAA TAC GAC TCA CTA TA GGG ACA ATT ACT ATT TAC AAT TAC A ATG GAC TAC AAA GAC GAC GAC GAT AAG AAG ACT ***YAC TGZ (XYZ)$_{18}$ YAC*** TGG TCA GCG AGC TGC **CAT CAT CAT CAT CAT CAT** CAC CGG CTA T (underlined bases represent the T7 RNA polymerase promoter; italic bases represent 21 consecutive random codons; boldface bases indicate the His6 tag.) (ii) The primers used for PCR amplification reaction were as follows: 5′ TTCTAATACGACTCACTATAG GGACAA TTACTATTTACAATTACA 3′ (sense), 5′ ATAGCCG GTGATGATGATGATGATGGC3′ (antisense). (iii) PCR reactions were performed in 25 μL volume containing 50 ng of DNA, 40 ng of primer, 200 μmol/L dNTPs, 2.5 mmol/L $MgCl_2$, 1xTaq buffer and 0.25 unit of Taq polymerase, and were incubated at 95℃ for 1 min, 55℃ for 1 min and 72℃ for 1.5 min for 25 cycles. At the end of the reaction, samples were incubated for an additional 10 min. The concentration of purified DNA was qualified by UV spectrometry.

2.2 *In vitro* transcription

The DNA library (500 ng) was used to generate large quantities of RNA using T7 RNA polymerase and the reagents included in the *in vitro* transcription kit. Once synthesized, the RNA was purified by electrophoresis on 5% TBE-urea polyacrylamide gel.

2.3 mRNA cross-linking reaction

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(i) Sequence of puromycin oligonucleotides: 5′ psoralen-TAGCCGGTGAAAAAAAAAAAAAAA-(PEG)2-ACC-puromycin 3′ .(ii) The 300 μL cross-linking reaction contained 60 μL of 500ng/μL RNA，30 μL of 5 μM Linker，30 μL of 250 μM Tris-HCl (pH7.4)，30 μL of 1 M NaCl（pH 7.5）. The linker was annealed to the target mRNAs by heating to 80℃ for 5 min followed by incubating at room temperature for 15 min. The reaction mixture was irradiated for 15 min at room temperature at 365 nm. The product was ethanol precipitated and resuspended in ddH2O.

2.4 *In vitro* translation and isolation of mRNA-protein fusion

Translation reactions were performed in rabbit reticulocyte lysate according to the manufacturer's specifications in 50 μL total volume at 30℃ for 1 h. By the end of the reaction, mRNA-protein fusion was induced by the addition of 10 μL of KCl (2.5 mol/L) and 3 μL of $MgCl_2$ (1 mol/L) with continued incubation at －20℃ overnight. The products were isolated by diluting approximately 10-fold into isolation buffer (100 mM Tris-HCl（pH 8.0）/10 mM EDTA/1 mol/L NaCl/0.1% Triton X-100) and incubated at 65℃ for 3-4 min. Supernatant was removed from the mixture by centrifugation at 5000 rpm for 10 min and transferred to a fresh tube containing oligo-dT cellulose (2-4 mg). Samples were rotated at room temperature for 1-2 h. The cellulose was then collected by centrifugation and washed with washing buffer (1:2 dilution of isolation buffer) three times, followed by elution with 50 μL ddH2O, and rotated for 1 h at room temperature to release the mRNA-protein fusion.

2.5 Generation of cDNA/mRNA-protein fusion

The cDNA/mRNA hybrid fusion product was generated using reverse transcription. The ethanol-precipitated samples was subjected to RT in a reaction (20 μL) containing 200 ng of antisense primer, 4 μL 5×first strand buffer, 4 μL 2.5 mM NTP, 1 μL RNasin（40 U/μL）and 3 μL AMV reverse transcriptase. The RT reaction was performed at 37℃ for 1 h.

2.6 Isolation of peptide fusions with immobilized TS30 RNA

(i) TS30 RNA represents the 5′ upstream binding site on TS mRNA, with the sequence 5′ CCG CCC GCC GCG CCA UGC CUG UGG CCG GCU-Bi 3′ .(ii) The biotinylated TS30 RNA was immobilized by mixing with a 50% slurry streptavidin agarose in 1×TE (pH 8.0) at a final RNA concentration of 5 μM for 1 h (25℃) with shaking. Immobilized RNA was washed and resuspended in 400 μL of binding buffer (10 mmol/L Hepes, pH 7.5, 0.5 mmol/L EDTA, 100 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.01% Nonidet P-40, 50 μg/mL yeast tRNA). Binding reactions were performed by adding 20 μL of the RT fusion followed by rotating at room temperature for 2 h. The agarose was precipitated by centrifugation and was washed 5-6 times with 100 μL of binding buffer (minus yeast tRNA). 1μL of RNase A (1 mg/mL) and Proteinase K (1 mg/mL) were added and incubated for 1 h at 37℃ to liberate bound molecules. The generation of cDNA could be amplified by PCR for another round of cycle.

3 Results and Discussion

3.1 dsDNA library

The starting library is constructed as a mixture of double-stranded DNA sequences. The DNA sequence contains several important design features [3]. A T7 promoter is present at the 5' end to allow synthesis of mRNA *in vitro*. The transcript begins with 3 G nucleotides to aid transcription initiation. A deletion mutant of the tobacco mosaic virus 5' is used to provide efficient translation initiation. The open reading frame and adjacent 3' constant region do not contain stop codons. The PCR amplification of library DNA was shown in figure 1.

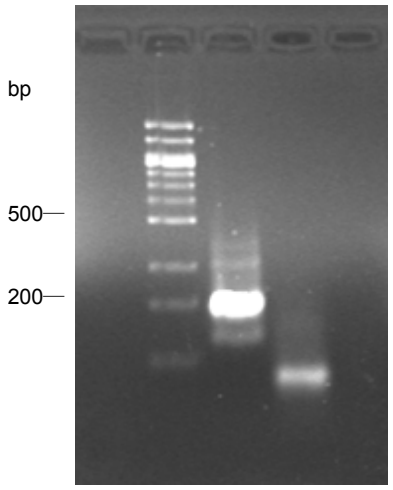

Fig. 1 Library DNA amplified by PCR. Lane 1: 100 bp DNA ladder; Lane 2: PCR products of library DNA; Lane 3: negative control

3.2 Synthesis of mRNA pool

Once generated, the dsDNA library was used to generate large

quantities of RNA enzymatically using T7 polymerase. The resulting RNA product was about 200nt (fig.2). Full-length RNA samples were then purified using urea PAGE.

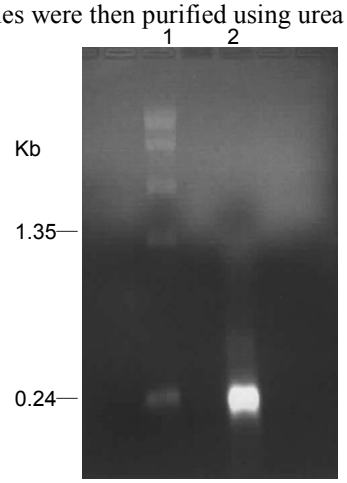

Fig. 2 *in vitro* transcription. Lane 1: RNA marker (0.24-9.5 Kb); Lane 2: library mRNA

3.3 Ligation of mRNA and linker

Ligation reaction are conducted with mRNA, DNA splint and puromycin oligonucleotide. Several features are important for efficient ligation. First, the splint should overlap both the 3' end of the mRNA and 5' end of the puromycin oligonucleotide by about 10 bases. Second, the mRNA template should be devoid of secondary structure at its 3' end as it can abolish ligation. Third, it is important to purify and desalt the template and linker. The result showed that the elution buffer containing NaOAc could obtain the better ligation effect(fig.3).

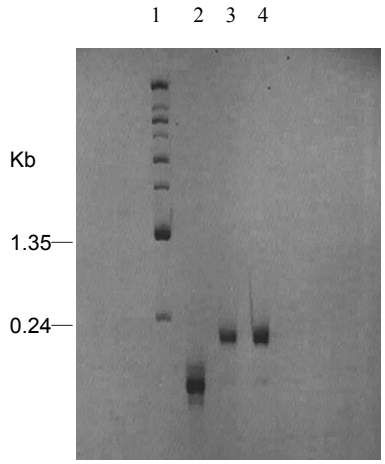

Fig.3 Purification of RNA with linker. Lane 1: RNA marker (0.24-9.5 Kb); Lane 2: library RNA alone; Lane 3: RNA-linker extracted by NaCl elution buffer; Lane 4: RNA-linker extracted by NaOAc elution buffer.

3.4 Formation of RNA-protein fusion

The cDNA/mRNA-linker-peptide fusion was generated using reverse transcriptase prior to the selective step. The amount of fusion product increased markedly after extended posttranslation incubation at low temperature, and Mg^{2+}, K^{+} were added. The cDNA/mRNA-linker-peptide had higher mobility than mRNA-linker-peptide fusion (fig.4).

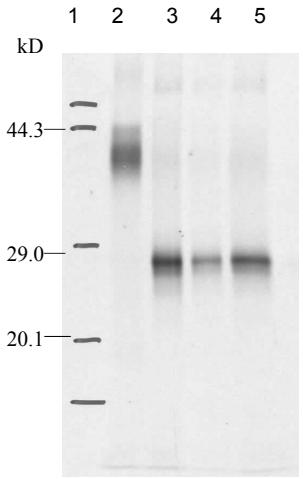

Fig 4 Fusion in the selection by SDS-page (10%). Lane 1 protein marker, low range. Lane 2, RNA-Linker-peptide after oligo dT purificaiton; Lane3, Library cDNA/RNA-Linker-peptide (after reverse transcription); Lane 4,5, fusion not bind with the agarose.

3.5 The selective step：Using an RNA target

The RNA-peptide fusion bound to its immobilized RNA target, TS30 RNA, the 5′ upstream protein binding site on human TS mRNA [4]. For selection experiment, the important thing is to control the specificity of binding. The highest level of selective enrichment was attained only when very large quantities of competitor were present in solution. Indeed, increasing the competitor (yeast tRNA and BSA) concentration dramatically increases the efficiency of selection（Fig.5）. Bound fusion was then eluted, and the eluted cDNA served as the template to generate a new library enriched for TS RNA binding proteins.

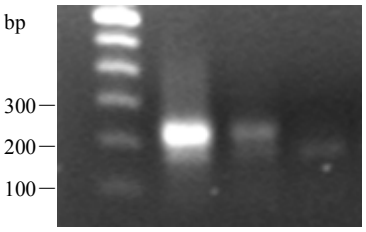

Fig.5 Isolation result of negative control (mRNA-protein fusion was incubated with the agarose gel, but no TS30 was included) Lane1：100

bp DNA ladder；Lane2：positive control, library DNA；Lane3：negative agarose gel, BSA and tRNA were not added to the gel，PCR product of the eluted cDNA；Lane4：negative agarose gel，BSA and tRNA were added to the agarose gel.

3.6 Identification of positive clones

PCR product from the final selection was purified and cloned into the pMD 18-T vector. Then the ligation products were transformed into *E. coli* DH5α. The positive clones were identified by blue-white colony and endonuclease digestion（Fig.6,7）, then could be picked randomly for sequencing.

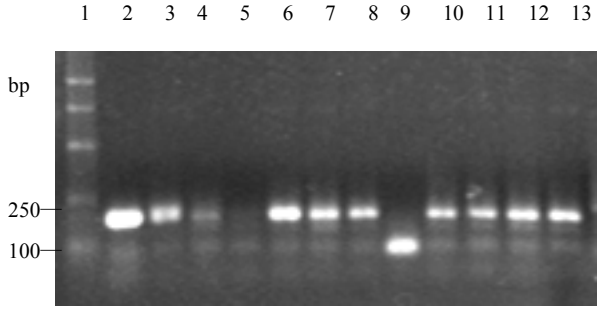

Fig. 6 Result of blue -white colony. Lane1: 100 bp DNA ladder; Lane2: positive control, PCR products of library DNA; Lane3, 4, 6, 7, 8, 10, 11, 12, 13: PCR products of positive clones; Lane5, 9: PCR products of negative clones.

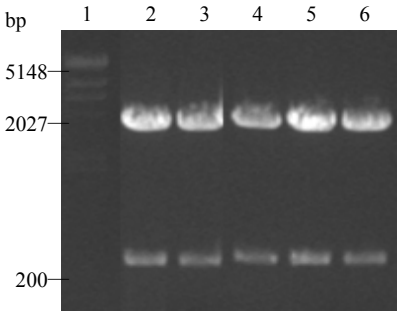

Fig.7 Identification of positive clones by endonuclease digestion. Lane1: marker; Lane 2, 3, 4, 5, 6: pMD 18-T simple vector digested with EcoR I, all of them are positive clones.

4 Conclusions

In vitro selection and directed evolution of peptide library are useful techniques for finding novel ligands that bind to target biomolecules [5]. In particular, mRNA display strategy, in which covalently linked conjugates of an mRNA and its peptide product are synthesized in an *in vitro* translation system, is advantageous for its large library size and the stable mRNA–peptide linkage. mRNA display has been used to select high affinity reagents from engineered libraries of ATP binding proteins [6], Jun-associated proteins [7], fibronectin-based intrabodies that detect and inhibit sars-cov N protein[8]， and single-domain antibody mimics [9，10]. We provide a detailed analysis of the critical steps of an mRNA-display-based selection, including the generation of an mRNA-displayed proteome library, formation of RNA-peptide fusions, and selection of proteins with desired functions. These results demonstrate that mRNA display technology will make powerful tool to discover new artificial ligands to target proteins or other biomolecules

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