

Direct Selection of cDNAs by Phage Display

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1. Introduction

Dozens of genomes will be partly or completely sequenced over the next few years. In the post genomic area, we are now facing the challenge of functionally characterizing thousands of genes generated by the genome projects. Selective enrichment of clones encoding a desired gene product and rapid handling of large numbers of individual clones resulting from screening of molecular libraries bear the potential to facilitate progress in this field. Phage surface display technology, first described by Smith (1), enables the construction of large combinatorial peptide and antibody libraries. The basic concept of phage display links the phenotype, expressed as fusion together with a phage surface coat protein, to its genetic information integrated into the phage genome. This procedure allows to survey large libraries for the presence of specific clones using the discriminative power of affinity selection (2-4). The selection of cognate phage (biopanning) is achieved by interaction between a solid phase-coated ligand and the phage library applied in fluid phase during multiple rounds of phage growth and selection. The field of phage display technology has developed rapidly, and antibodies, enzymes, enzyme inhibitors, hormones, DNA binding molecules, and allergens have been successfully selected from molecular libraries (5-11). These examples clearly demonstrate the general applicability of linking genotype and phenotype to phage coat proteins. Molecular libraries allow the rapid identification of peptide-ligand interactions. In combination with high-throughput screening technology, they will play an important role in a rational approach for the identification of gene products (12,13). However, one of the limitations of filamentous phages as display vectors for cDNA libraries is a direct consequence of the capsid structure. The integrity of the carboxy terminus of both pIII and pVIII, the phage coat proteins mostly used in phage display (14), is essential for phage assembly and hampers insertion of foreign peptides. Therefore, insertion of heterologous genes into pIII or pVIII to generate fusion proteins can only be tolerated at the amino terminus. Translational stop codons present at the 3' end of nontranslated regions of eukaryotic mRNA prevent, however, construction of fusion proteins N terminal to the pIII and pVIII phage coat proteins (15,16). In contrast, the minor coat protein pVI has been shown to tolerate insertions at its C terminus and has been proposed as cDNA display system to overcome this limitation (17).

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However, the filamentous phage vector most used for construction and screening of cDNA libraries is based on an indirect fusion strategy, in which cDNA inserts fused to the 3' end of the Fos leucine gene are co-expressed with a gene encoding a Jun leucine zipper fused N terminal to pIII (**13,15,16,18**). The high affinity between the Jun and Fos leucine zippers (**19**) efficiently links the cDNA gene products outside of the phage capsid. Thus, the fusion products become solvent exposed, and phage displaying any given gene product can be affinity purified from nonbinding phage by interactive panning cycles against immobilized ligands (**16,18**). Although most of the cDNA libraries constructed and screened using the pJuFo vector were devoted to the isolation of IgE binding molecules from complex allergenic sources (**13**), many other applications have been reported (**20–23**), demonstrating the versatile applicability of the cloning system. In particular, it has been demonstrated that pJuFo cloning strategy can be used to select ligands for receptors expressed in differentiated U937 cells (**23,24**). Therefore, the technology might be potentially applicable to select gene products able to interact with whole cells during different time points of differentiation. In this chapter, we describe a procedure for the direct generation of cDNA phage surface display libraries starting from isolated mRNA.

2. Materials

1. pJuFo II Vector: free, available from nonprofit research organizations.
2. cDNA synthesis kit for construction of directional libraries (Stratagene, La Jolla, CA, cat. no. 200401).
3. DNA restriction and modifying enzymes (Roche Diagnostics, Mannheim, Germany): *EcoRI* (cat. no. 703737), *XhoI* (cat. no. 899194), calf intestinal phosphatase (CIP) (cat. no. 713023), T4 DNA-ligase (cat. no. 481220).
4. *Escherichia coli* XL1-Blue strain (cat. no. 200301), VCSM13 helper phage (cat. no. 200251), and premade λ -ZAP libraries (Stratagene).
5. Media:
 - a. Super broth (SB): 30 g tryptone, 20 g yeast extract, 10 g MOPS per liter, pH adjusted to 7.0 with HCl. Autoclave at 121°C.
 - b. Luria-Bertani medium (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH adjusted to 7.5 with NaOH. Autoclave at 121°C.
 - c. LB agar: make up liquid LB medium and, just before autoclaving, add 15 g agar per liter.
6. RNA and plasmid purification kits Qiagen (Hilden, Germany): Oligotex direct mRNA kit (cat. no. 72041), Qiagen plasmid mini kit (cat. no. 12123), Qiagen plasmid maxi kit (cat. no. 12162).
7. Solutions:
 - a. Tris-buffer (1 M): dissolve 121.1 g Tris (Fluka, Buchs, CH, cat. no. 93362) in 800 mL of H₂O. Adjust the pH to 7.4 by adding concentrated HCl (Fluka, cat. no. 84411), add H₂O to 1 L, and autoclave at 121°C.
 - b. EDTA (0.5 M): add 185.1 g of disodium ethylene diamine tetraacetate (Fluka, cat. no. 03609) to 800 mL H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH pellets (Fluka, cat. no. 71687), add H₂O to 1 L, and autoclave at 121°C.
 - c. Tris-EDTA (TE): mix 10 mM Tris (pH 7.4) and 1 mM EDTA (pH 8.0).
 - d. Tris-acetate EDTA (TAE): prepare a 50X concentrated stock solution composed of 242 g Tris, 57.1 mL glacial acetic acid (Fluka, cat. no. 45732), and 100 mL 0.5 M EDTA (pH 8.0). Add H₂O to 1 L.

Selective Cloning of cDNAs

- e. Phosphate-buffered saline (PBS): 50 mM sodium phosphate, pH 7.4, (Fluka, cat. no. 04278), 150 mM NaCl. Autoclave at 121°C.
- f. Tris-buffered saline (TBS): 50 mM Tris, pH 7.5, 150 mM NaCl, sterilize by filtration.
- g. NaOAc (3 M): Dissolve 408.1 g of sodium acetate (Fluka, cat. no. 71183) in 800 mL of H₂O. Adjust pH to 5.2 with glacial acetic acid, the vol to 1 L, and autoclave at 121°C.
- h. Blocking buffer: 2% bovine serum albumin (BSA) (Fluka, cat. no. 05491) in PBS, pH 7.4.
- i. Phage elution buffer: 0.1 M HCl, pH 2.2, adjusted with solid glycine (Fluka, cat. no. 50049), 1% BSA.
8. Electroporation apparatus, cuvettes, and electroporation protocols (Bio-Rad, Hercules, CA).
9. Polyethylene glycol (PEG)-NaCl solution: to 200 g PEG 6000 (Fluka, cat. no. 81260) and 146.1 g NaCl, add ddH₂O to 1 L, autoclave at 121°C.
10. For bacterial plasmid minipreps, we use Miniprep Express Matrix (BIO 101, Vista, CA, cat. no. 2000-200).
11. Ampicillin (Amp) (Fluka, cat. no. 10047) stock solution 100 mg/mL in H₂O. Sterilize by filtration and store in aliquots at -20°C.
12. Tetracyclin (Tc) (Fluka, cat. no. 87130) stock solution 12.5 mg/mL in ethanol-H₂O (50% v/v). Store at -20°C in the dark.
13. Kanamycin (Kan) (Fluka, cat. no. 60615) stock solution 25 mg/mL in H₂O. Sterilize by filtration and store in aliquots at -20°C.

3. Methods

3.1. Vector Preparation

1. Spread *E. coli* XL1-Blue cells harboring pJuFo II on LB agar plates containing 100 µg/mL Amp and 12.5 µg/mL Tc (LB^{Amp,Tc}) to obtain single colonies.
2. Pick a single colony with a sterile loop, transfer to a sterile tube containing 5 mL LB^{Amp,Tc} medium and grow overnight.
3. Inoculate 500 mL LB^{Amp,Tc} medium with the overnight culture from **step 2** and grow at 37°C, 220 rpm, until culture is stationary.
4. Collect bacteria by centrifugation (10 min at 4000g at 4°C).
5. Lyse cells and isolate plasmid DNA according to the Qiagen MAXIprep protocols. Determine DNA concentration and purity by gel electrophoresis and store at -20°C until use.
6. Set up restriction digests as follows:

Purified pJuFo II DNA	10 µg
10X Restriction buffer	10 µL
<i>EcoRI</i>	30 U
<i>XhoI</i>	30 U
ddH ₂ O to	100 µL
CIP	0.5 U

Digest at 37°C for 3 h. Add another 3 U of CIP and incubate for 10 min at 37°C.

7. Run restricted DNA on 1.0% agarose gel (2 µg DNA/lane at 80 V), excise linearized pJuFo II vector (approx 4.3 kb), and purify the band using a commercial silica based kit (*see Note 1*) or by electroelution (25).
8. Ethanol precipitate the eluted DNA by adding 1/10 vol 3 M NaAc, pH 5.2, and 2.5 vol EtOH, store at least 2 h at -20°C.

9. Collect DNA by centrifugation in a microcentrifuge (30 min, $>10,000g$ at 4°C), air-dry pellet, and resuspend linearized DNA in $40\ \mu\text{L}$ TE buffer, store at -20°C until use.
10. To test the vector self-ligation, set up a ligation with the linearized vector ($0.1\ \mu\text{L}$ vector, $1\ \mu\text{L}$ T4 DNA ligase, $1\ \mu\text{L}$ 10X ligation buffer, ddH_2O to a total volume of $10\ \mu\text{L}$ and incubate 4–14 h at room temperature). Pulse $100\ \mu\text{L}$ competent *E. coli* XL1-Blue cells (26) (see Note 2) at 2.5 kV, 25 μF , and 200 Ohm. Flush cuvette immediately after electroporation with 1 mL prewarmed (37°C) SB medium and incubate 1 h in a microcentrifuge tube at 37°C with shaking (200 rpm). Spread 10 and $100\ \mu\text{L}$ of the transformants on $\text{LB}^{\text{Amp.Tc}}$ plates, incubate at 37°C overnight, and determine the number of transformants (see Note 3).

3.2. Preparation of cDNA Inserts

Alternatively to the following protocol, premade commercially available λ -ZAP libraries (Stratagene) can be used to prepare *EcoRI/XhoI*-restricted cDNA inserts (16).

1. Isolate poly(A)⁺ mRNA from a source of choice using a standard procedure. Reliable results are obtained with the Qiagen Oligotex direct mRNA kit (see Note 4).
2. Generate cDNA using the Stratagene cDNA synthesis kit.
3. Digest the cDNA with *EcoRI* and *XhoI* and run the restriction mixture on a 1.0% agarose gel. Cut out inserts exceeding 500 bp in length (see Note 5).
4. Purify size-selected cDNAs according to Subheading 3.1., steps 7–9.

3.3. Generation of cDNA Phage Surface Display Libraries

1. Set up test ligations to determine the best vector/cDNA insert ratio:

Linearized pJuFo II DNA	250 ng
10 $\times$ ligase buffer	1 μL
T4 DNA ligase (>1 Weiss U)	1 μL
cDNA inserts	10, 50, and 100 ng
Water to an end volume of	20 μL

Ligate for at least 4 h at room temperature or overnight.

2. Electroporate ligation mixture into competent *E. coli* XL1-Blue cells (pulse at 2.5 kV, 0.2-cm gap cuvettes, 25 μF , and 200 Ohms) flush the electroporation cuvette immediately with 1 mL prewarmed SB medium, and plate on $\text{LB}^{\text{Amp.Tc}}$ plates to determine the optimal vector:insert ratio.
3. For construction of a library, scale up ligation using the optimal vector:insert ratio determined in step 2 to obtain a library size of $>10^7$ colony forming units (cfus).
4. Heat-inactivate the ligation mixture (10 min at 60°C). Precipitate DNA by adding 1/10 vol of 3 M NaAc (pH 5.2), 1/200 vol of glycogen (20 mg/mL), and 2.5 vol. of EtOH (>2 h at -20°C). Collect DNA by centrifugation (30 min at $>10,000g$), air-dry the pellets, and resuspend in 20 mL ddH_2O .
5. Electroporate *E. coli* cells as in step 2. Flush the electroporation cuvette immediately with 1 mL prewarmed SB medium, then with additional 2 mL SB medium, transfer to a 50-mL conical tube, and incubate immediately for exactly 60 min at 37°C with shaking at 200 rpm (see Note 6).
6. Add SB medium (Amp 20 $\mu\text{g/mL}$) prewarmed at 37°C to a vol of 10 mL and immediately titer the transformants by plating 10, 1, 0.1, and 0.01 μL on $\text{LB}^{\text{Amp.Tc}}$ plates. Incubate plates overnight at 37°C and calculate the primary size of the library from the number of transformants obtained from the dilutions plated.
7. Incubate the 10-mL culture for 60 min at 37°C at 250 rpm.

Selective Cloning of cDNAs

8. Add Amp to a final concentration of 50 $\mu\text{g/mL}$ and incubate for an additional 1 h at 37°C at 250 rpm.
9. Add helper phage VCSM13 (10^{12} plaque forming units [pfu]) and adsorb 5 min at 37°C (see Note 7).
10. Adjust the culture volume to 100 mL with SB medium (Amp 100 $\mu\text{g/mL}$, Tc 12.5 $\mu\text{g/mL}$) prewarmed at 37°C and incubate on a shaker (250 rpm at 37°C for 2 h).
11. Add Kan to a final concentration of 70 $\mu\text{g/mL}$ and incubate overnight (250 rpm) at 30° or 37°C (see Note 7).
12. Spin down cells (5000g at 4°C for 15 min). Place the supernatant in a clean bottle and add 1/5 vol cold PEG-NaCl solution.
13. Place the bottle in an ice-water bath for 60 min to precipitate phage.
14. Spin down (8000g at 4°C for 20 min) and discard supernatant. Allow the bottle to drain on a paper towel (5 min) to remove as much PEG as possible.
15. Resuspend pellet in 2 mL TBS containing 1% BSA, clean by microcentrifugation (2 min at $>10,000\text{g}$), transfer the supernatant to a new tube, and store at 4°C; for long-term storage, store at -20°C.
16. Titer the phage suspension by infecting 200- μL aliquots of freshly grown *E. coli* XL1-Blue cells ($A_{600\text{ nm}} = 0.5$) with 1 μL of 10^{-8} , 10^{-9} , 10^{-10} , and 10^{-11} phage dilutions in LB medium for 15 min at room temperature and plate on LB^{Amp,Tc}. Incubate plates overnight at 37°C to visualize transfectants.

3.4. Testing the Complexity of the Library

1. To test the successful generation of a library, pick 20 colonies from the titrating plates, grow in 3 mL LB^{Amp, Tc} medium, and make plasmid minipreps using the Miniprep Express Matrix kit.
2. Digest minipreps with *EcoRI/XhoI* and visualize the size distribution of the inserts on agarose gels. Inserts should be of variable size (see Note 8).
3. Alternatively, presence and distribution of inserts can be tested by polymerase chain reaction (PCR), starting directly from colonies picked from the titrating plates (see Note 9).

3.5. Enrichment of Phage with Affinity for a Ligand

This section deals with antigens or antibodies used as ligands to enrich phage by affinity interaction. For the use of other ligands see Note 10.

1. Coat 2 to 3 wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate with 150 μL antigen or antibody solution (10 $\mu\text{g/mL}$). Choose a coating buffer suitable for your protein. Use PBS, pH 8.0, if working with monoclonal antibodies. Coating may be performed at 37°C for 1 h or at 4°C overnight.
2. Wash twice with PBS, pH 7.4. Block by filling the wells completely with blocking buffer, seal the plate, and incubate for 1 h at 37°C, then wash twice with PBS.
3. Add 10^{11} cfus of phage from the cDNA library (generally 50 μL) to each well, seal the plate, and incubate for 2 h at 37°C.
4. Remove phage, wash each well once with water. Wash 10 times with TBS containing 0.5% Tween-20, 10 times with TBS, and twice with distilled water at room temperature. Pipet up and down several times to remove nonspecified phage, fill completely with washing buffer (see Note 11). Elute phage with 150 μL phage elution buffer. Incubate for 10 min at room temperature.
5. Remove the eluate by pipetting up and down vigorously and transfer immediately to a tube containing 18 μL 1 M Tris (pH 8.0) per 150 μL eluate to neutralize.

6. For further rounds of panning, use eluted phage to infect 4 mL of freshly grown *E. coli* XL1-Blue cells ($A_{600\text{ nm}} = 0.5$) for 15 min at 37°C.
7. Add prewarmed (37°C) SB medium (Amp 20 µg/mL, Tc 12.5 µg/mL) to 10 mL. Immediately plate 20, 10, and 1 µL on LB agar to determine the number of packaged phagemids. Incubate plates overnight at 37°C and count colonies.
8. Incubate the culture for 1 h at 37°C, 250 rpm, and repeat **Subheading 3.3., steps 7–16**.
9. Perform successive rounds of affinity enrichment, repeating **steps 1–6**. If phage that are able to interact with the ligand are present in the library, the number of phage eluted from the wells (**step 5**) will increase with the rounds of panning. Determine the percentage yield of phage (% yield = [Number of phage eluted/Number of phage applied] × 100) for each round of panning. If no enrichment is seen in the following 5 rounds of panning, the desired clone is not likely to be isolated from the library, and the search should be concluded (*see Note 12*).
10. Enrichment of specific cDNAs can also be detected by PCR amplification directly from phage samples obtained after each round of panning (*see Note 13*).
11. If phage enrichment is successful proceed with subcloning of the inserts into a suitable vector to produce recombinant protein or with sequence determination to identify the encoded products (*see Note 14*).

4. Notes

1. In our hands, GeneClean and Nucleotrap glassmilk kits from Bio 101 and Machery-Nagel (Dürren, Germany) work efficiently for the isolation of cDNA inserts from agarose gels.
2. We normally produce electrocompetent cells in our laboratory. However, the transformation efficiency for construction of cDNA libraries should be $>1 \times 10^8$ cfus/µg of transformed plasmid DNA and needs to be tested for each new batch of cells. Commercially available competent cells are warranted in terms of transformation efficiency.
3. A low vector background is extremely important for the construction of cDNA libraries displayed on phage surface, because empty phagemids generated during superinfection with helper phage can overgrow specific phage during successive rounds of panning. The background of the vector can be reduced by treatment of the restricted DNA with alkaline phosphatase. Dephosphorylation of the vector may reduce the efficiency of ligation but is, however, strongly recommended. More than two to three colonies/plate for 100 µL transformation mixture cannot be tolerated.
4. Any cDNA library depends primarily on the quality of the mRNA used for cDNA synthesis. Make sure that methods or kits used for mRNA isolation work properly. Directional *EcoRI/XhoI* cloning of cDNAs generated by the ZAP cDNA synthesis kit into the modified pJuFo II vector takes advantage of the methylation of internal restriction sites in the cDNAs, enhancing the quality of the libraries.
5. Separation of small cDNAs from larger cDNA inserts on agarose gels is always incomplete. Therefore, inserts of >500 bp will be present in the library in spite of the cutoff.
6. The electroporation step is crucial for the quality of the library and should be done very carefully. The ligation mixture can be transformed in several portions to reduce the risk related to electroporation. Sometimes electroporation is not successful due to accidental discharging of the condensor, which results in the well known electrotransformation spark and the complete killing of the cells.
7. We use the Kan-resistant helper phage VCSM13 for superinfection. However, helper phage R408 works as well as VCSM13. If R408 helper phage is used, addition of Kan should be omitted. To generate a good phage surface displayed library, *E. coli* cells should be superinfected with helper phage at a multiplicity of infection of 10.

Selective Cloning of cDNAs

8. The diversity of a library can be defined as the percentage of different clones contained in the library. Therefore, a high percentage of inserts differing in size, together with the absence of empty clones, warrant a good quality of the library. Since the expected diversity is in the range of 10^6 to 10^9 independent clones, this experiment gives only a glance of the diversity of the library. Screening of a statistically significant number of colonies for an exact determination of the diversity is limited by practical reasons.
9. PCR amplification primers and conditions. To directly scan single clones and/or the whole phage repertoire the following PCR primer set allow visualization of the insert distribution and direct subcloning into the high level expression vector pQE30 (Qiagen, Hilden). The two primers are flanking the cDNA construct, priming at the 5' end on the Fos gene and at the 3' end on the vector. The 3' primer contains a *Hind*III restriction side for direct subcloning of the PCR product into pQE30 as *Bgl*II/*Hind*III fragment.
5' JuFo: 5'-AAAGAAAAGCTGGAGTTCATCCTGGC-3'
3' JuFo: 5'-GGCCAGTGAATTGTAATACGACCCCAAGCTTGGG-3'
PCRs are carried out at 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s, for 30–35 cycles in the PCR amplification mixture containing 2.5 mM Mg^{2+} . As target for PCR amplifications, it is possible to directly transfer bacteria from single colonies to tubes containing the PCR mixture. However, to obtain reproducible results, we recommend to use 1 μ L of 1/400 diluted plasmid minipreps as PCR targets.
10. Many different ligands have been successfully used to screen cDNA libraries displayed on phage surface (13). In addition to mass law considerations (27), two important points have to be taken into account: (1) the cDNA molecules obtained after reverse transcription are thought to contain the information for all those proteins that were expressed in the source at the time of mRNA isolation. It is, therefore, crucial to ensure that the desired gene product is actually expressed in the source chosen for mRNA extraction; and (2) we draw attention to the fact that many ligands, when immobilized directly to solid phase, might become altered loosing their biological functionality. A ligand used to screen phage libraries should be tagged or immobilized to solid phase in such a way that allows retention of its ability to interact with the wanted target.
11. To avoid contamination of pipets and subsequent cross-contaminations, we always use aerosol-resistant tips for all steps in phage handling, especially for the washing procedure. For an excellent theoretical review about the factors influencing the probability of selecting phage able to interact with the ligand, including washing steps, see ref. 27.
12. Enrichment depends on many factors including the quality of the library, the affinity of the ligands for the target, and the number of specific ligands available for a specific target. Therefore, enrichment of libraries with biologically active homogeneous ligands are likely to be successful if the interacting gene product is present in the library. When using complex ligands like human serum, success will depends on the percentage of the specific ligand (e.g., antibody) present in the serum used. It is almost impossible to select specific phagemids from background if binding of a wanted gene product to its specific ligand is limiting compared to the unspecific binding (27).
13. Phage PCR is carried out with the same primer set and conditions as described in Note 6, but with a diluted phage solution (approx 10^{11} cfu) as target. To visualize the enrichment, 10 μ L of the PCR products are loaded on a 1.2% agarose gel. The size distribution of the PCR mixture is condensing from a smear in early rounds to more intense discrete bands in later rounds of successful enrichment. Prominent PCR bands are likely to represent enriched genes and can be directly excised from the gel for subcloning, sequencing, and protein production.
14. Low amounts of protein derived from inserts of putative clones can be produced as secreted protein by isopropyl- β -D-thiogalactopyranoside (IPTG) (2 mM) induction of the *Lac*

promoter controlling Fos-expression in absence of helper phage. Alternatively, phage derived from single clones can be directly used in phage ELISA experiments or Western blot analyses as described previously (15). If large amounts of protein are required, subcloning in high-level expression vectors is required.

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Selective Cloning of cDNAs

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