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Cloning Sequence-Specific DNA-Binding Factors from cDNA Expression Libraries Using Oligonucleotide Binding Site Probes

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1. Introduction

The method described in this chapter has been used in the molecular cloning of transcription factors and other factors with DNA-binding activity toward specific double-stranded DNA sequences. The protocol is based on the method of Singh et al. *(1)* and shares feature with the immunological approach to screening cDNA expression libraries (*see* Chapter 13). The principle is to probe a cDNA expression library (usually a λ-phage expression library) with a labeled double-stranded DNA probe containing the sequence recognized by the factor in question. Recombinants expressing a protein capable of binding the probe sequence in the presence of nonspecific competitor DNA are thus identified and can be isolated.

The most commonly used vectors for the construction of cDNA expression libraries are the bacteriophage vectors λgt11 (Huynh et al. *[2]*) and λ ZAP (Stratagene, La Jolla, CA). In both of these vectors, cDNA inserts are cloned into the coding region of the *LacZ* gene. In the case of λgt11, the cloning site is just upstream of the *LacZ* translational stop signal and in λ ZAP, which employs *LacZ* α complementation for blue/white selection of recombinants, cDNAs are cloned into the *LacZ* α peptide-coding sequence. Thus, in both vectors, the cloned cDNAs are potentially expressed as β-galactosidase fusion proteins (*see* Note 1). Problems of underrepresentation of cDNAs encoding polypeptides that are toxic or that reduce cell growth are minimized by the use of host cells expressing the *Lac* operon repressor to prevent *LacZ*-directed expression of the fusion protein until plaque formation is visibly under way.

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Fusion protein production is then induced for a few hours with isopropyl β-D-thiogalactopyranoside (IPTG), which inactivates the *Lac* repressor. Using λ-phage expression libraries, the use of DNA probes has been particularly successful in the cloning of members of the leucine zipper family of transcription factors *(1,3–6)*, and has also been used for other classes of DNA-binding protein *(7,8)*. The following protocols where written for λgt11 libraries *(2)*, but these methods can be employed with λ ZAP libraries (Stratagene) with minor modifications (*see* Note 1).

There are several notes and cautions that should be considered before embarking on library screening. First, if posttranslational modification, such as phosphorylation or specific proteolytic cleavage, is required for efficient DNA-binding activity, then the factor may not bind DNA with sufficiently high affinity when expressed in *Escherichia coli*. Careful selection of binding and washing conditions may circumvent this problem. Poor solubility or incorrect protein folding of a bacterially expressed factor may preclude efficient DNA binding. However, DNA-binding activity can, at least in some cases, be regenerated by denaturation with guanidinium chloride and gradual renaturation (*see* Section 3.5.). Finally, a problem with binding site screening arises if the factor of interest binds DNA efficiently only as a heterodimeric or heteromeric complex, since only a single polypeptide sequence can be expressed from any one clone. Similarly, this method is not appropriate for the cloning of components of a multisubunit transcription factor complex if they do not stably bind DNA themselves in the absence of the other components. South-western blotting can be used to determine whether DNA-binding activity resides in a single polypeptide (*see* Note 2). South-western blotting is a good preliminary experiment to carry out before attempting to screen libraries. A positive South-western result not only indicates that DNA-binding activity resides in a single polypeptide, but also proves that DNA binding activity is maintained when the protein is bound to a nitrocellulose membrane.

The method discussed herein is split into several parts: library titering, library plating and making replica lifts, probe labeling and screening, and finally plaque purification. In addition, an optional step that can improve the signal from some factors is included in Section 3.5.

2. Materials

2.1. Titering the Library

1. Expression cDNA library in λgt11 or λ ZAP (*see* Note 1)
2. Fresh plate of *E coli* Y1090 maintained on Luria-Bertoni (LB) medium agar containing ampicillin at 50 μg/mL (for λ ZAP libraries, use *E coli* XL1-Blue [*see* Note 1] maintained on LB agar containing tetracycline at 12.5 μg/mL).

3. LB medium containing 10 m*M* $MgSO_4$· 10 g tryptone, 5 g yeast extract, 10 g sodium chloride/L. Sterilize by autoclaving, and add $MgSO_4$ to 10 m*M* from a filter-sterilized 1*M* stock
4. LB agar. 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, 15 g bacteriological agar/L Sterilize by autoclaving, and add $MgSO_4$ to 10 m*M* from a filter-sterilized 1*M* stock.
5. Well-dried LB agar 9-cm diameter plates.
6. Autoclaved top agarose· 0.7 g agarose in 100 mL LB medium Melt in microwave, and maintain at 45°C for 20 min prior to use.
7. Maltose solution 10% (w/v) in water. Autoclave
8. SM buffer to dilute phage· 100 m*M* NaCl, 10 m*M* $MgSO_4$, 50 m*M* Tris-HCl, pH 7.5, 0.01% gelatin. Autoclave
9. Freshly streaked LB agar plate of *E coli* Y1090 (or a fresh plate of *E coli* XL1-Blue [Stratagene] if using λ ZAP; XL1-Blue cells should be cultured on LB agar containing tetracycline at 12.5 μg/mL).
10. Falcon 2063 tubes (Becton Dickinson)

2.2. Library Plating and Replica Lifts

1. Titered λgt11 or λ ZAP expression library (*see* Sections 2.1. and 3 1 and Note 1)
2. 10–20 Well-dried LB agar 15- and 9-cm diameter plates.
3. Autoclaved top agarose· 0.7 g agarose in 100 mL LB medium. Melt in microwave and maintain at 45°C for 20 min prior to use.
4. Maltose solution 10% (w/v): Sterilize by autoclaving.
5. SM buffer to dilute phage· 100 m*M* NaCl, 10 m*M* $MgSO_4$, 50 m*M* Tris-HCl, pH 7.5, 0.01% gelatin. Autoclave.
6. Fresh plating cells (*see* Section 3.1.1 and Note 1).
7. Nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). 132- and 82-mm circles.
8. IPTG: 1*M* stock in water. Store at –20°C.
9. Wash buffer: 50 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 0.05% lauryl dimethylamide oxide (LDAO; Calbiochem, La Jolla, CA), 1 m*M* dithiothreitol (DTT) (added just before use).
10. South-western block: 2.5% dried milk powder, 50 m*M* HEPES, pH 8.0, 10% (v/v) glycerol, 50 m*M* NaCl, 0.05% LDAO, 1 m*M* DTT (added just before use).
11. Falcon 2059 tubes (Becton Dickinson).

2.3. Probe Labeling

2.3.1. Oligonucleotide Annealing

1. T4 polynucleotide kinase buffer (10X): 500 m*M* Tris-HCl, pH 7 5, 100 m*M* $MgCl_2$, 5 m*M* DTT, 1 m*M* spermidine. Make up freshly.
2. Complementary single-stranded binding site oligonucleotides in TE or water at approx 1 mg/mL. Store at –20°C (*see* Note 4).
3. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.

2.3.2. Probe Labeling and Ligation

1 T4 polynucleotide kinase and 10X polynucleotide kinase (PNK) buffer 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 5 m*M* DTT, 1 m*M* spermidine Make up freshly
2 [γ-^{32}P]-Adenosine triphosphate (ATP) at 3000–5000 Ci/mmol.
3 Sephadex G-50 M (Pharmacia, Uppsala, Sweden) autoclaved in water or TE
4 T4 DNA ligase and 10X ligase buffer: 500 m*M* Tris-HCl, pH 7 5, 100 m*M* $MgCl_2$, 10 m*M* DTT, 10 m*M* ATP, 10 m*M* spermidine.

2.4. Screening and Selection of Positive Plaques

1. Poly(dA)/Poly(dT) (Pharmacia) stock at 1 mg/mL made up in water.
2 TNE-50· 50 m*M* NaCl, 10 m*M* Tris-HCl, pH 7 5, 1 m*M* EDTA, 1 m*M* DTT. Add DTT just before use and store buffer at 4°C.
3 South-western block (*see* Section 2.2)

2.5. Guanidinium Denaturation and Renaturation

1. TNE-50 (*see* Section 2.4.).
2. Guanidinium chloride (6*M*) in TNE-50.
3. South-western block (*see* Section 2 2.).

3. Methods

3.1. Titering the Library

Titering of a library phage stock is critical. It allows the calculation of the number of phage particles or plaque forming units (PFU) in a given volume of stock A known density of PFU can then be plated out for screening

3.1.1. Preparation of Plating Cells

1. Make fresh plating cells by inoculating one colony into 20 mL of LB medium containing 0 2% maltose. For all λ work, use LB medium containing 10 m*M* $MgSO_4$.
2 Shake the culture vigorously at 37°C until stationary phase is reached (overnight incubation, for example).
3. Pellet the cells, and resuspend in half their original volume using SM buffer Plating cells may be stored on ice until required (up to 24 h).

3.1.2. Plating

1. Make several 10-fold serial dilutions of the library phage stock in 100-µL aliquots of SM in Falcon 2063 tubes
2. Add 300 µL of fresh plating cells to each tube, gently mix, and place the tubes in a water bath at 37°C for 15 min for infection to occur.
3 Add 3 mL molten top agar at 45°C to each tube, and pour onto a series of prewarmed 90-cm LB agar plates.
4 When the top agar has set, transfer the plates to a 42°C incubator (37°C for λ ZAP) and incubate overnight.

5 Count the number of plaques formed on one of the plates to calculate the titer of the original phage stock

3.2. Library Plating and Replica Lifts

Once the titer has been ascertained, the library can be plated on 15-cm plates, and protein expression induced for transfer to nitrocellulose membranes. Once again the protocol assumes the use of a λgt11 library and *E. coli* Y1090 plating cells *(2)*, but only minor modification is required for λ ZAP libraries (*see* Note 1).

1 Having determined the titer of the library, plate out at least 10^6 PFU at a density of up to 10^5 PFU/15-cm plate (Note 3). For each plate, dilute phage into 200 µL of SM buffer, and add 600 µL of fresh plating cells (*see* Section 3.1.1.) using tubes, such as Falcon 2059 tubes Leave to infect for 15 min at 37°C, and then add 7.5 mL molten top agarose and pour onto prewarmed LB-agar plates.
2 Leave the plates at room temperature for the top agar to set, and transfer to a 42°C incubator until plaques are just visible as pinpricks on the bacterial lawn. This takes 3–4 h. (For λ ZAP, incubate at 37°C for 6–8 h or overnight.)
3 Soak one nitrocellulose filter for each plate in 10 m*M* IPTG and dab off the excess liquid. Working quickly, take one plate at a time, place a numbered filter, still damp with IPTG solution, on the surface of the top agar and then transfer the plate to a 37°C incubator. Incubate for 1–2 h. It is best to handle one plate at a time as the temperature of the agar should not drop below 37°C for λgt11 libraries.
4. Mark the position of each filter relative to the plate by piercing the filter and agar with three to four orientation holes using a syringe needle dipped in India ink Using blunt-ended forceps, carefully remove the filter and transfer to a plastic box (approx 20 × 20 × 5–10-cm deep) containing 500 mL of wash buffer
5. Place a second IPTG-soaked filter on each plate in turn, and incubate at 37°C for a further 2–3 h, again marking the filters for alignment before removing from the plates. For the second lifts, a permanent marker pen can be used to make marks on the filters over the syringe needle holes that should be visible in the agar. Use a light box to make this easier if necessary
6. Remove the second set of filters to wash buffer. All filters should be washed for 5–10 min to remove loose pieces of top agarose and so reduce background. The filters are now ready for blocking (*see* Section 3.4.).
7. Once the second set of filters have been removed, wrap the plates in Saran Wrap™ or equivalent, and store them at 4°C until positive plaques have been identified.

3.3. Probe Labeling

The selection of the probe is another important feature and is discussed in more detail in Note 4. The author has used a specific factor binding sequence in a 20- to 25-mer double-stranded oligonucleotide with *Hin*dIII

sticky ends to allow efficient labeling and concatenation. The probe sequence is first annealed from single-stranded oligonucleotides, and the double-stranded oligonucleotide is then labeled with [γ^{32}P]-ATP using T4 PNK and concatenated by end to end ligation with T4 DNA ligase.

3.3.1. Oligonucleotide Annealing

1 Take 40 µg each of the upper and lower strand oligonucleotides made up in water, and place in a 1.5-mL microfuge tube Add 10 µL of 10X T4 PNK buffer (500 m*M*, Tris-HCl, pH 8.0, 100 m*M* $MgCl_2$, 5 m*M* DTT, 1 m*M* spermidine) Make the volume up to 100 µL with deionized water.
2 Incubate at 90°C for 5 min, 65°C for 10 min, 37°C for 15 min, and then at room temperature for 30 min.
3 Ethanol-precipitate, and take the double-stranded oligonucleotide up in TE at 100 ng/µL
4. Store the double-stranded oligonucleotide at –20°C until required.

3.3.2. Probe Labeling and Ligation

1 In a 1.5-mL microfuge tube mix 4 µL double-stranded oligonucleotide (0 1 µg/µL), 2 µL 10X polynucleotide kinase buffer, 5 µL (50 µCi) [γ^{32}P]-ATP (5000 Ci/mmol), 2 µL (20 U) T4 polynucleotide kinase, 7 µL H_2O.
2 Incubate at 37°C for 1 h.
3 Add 80 µL of TE to the labeling reaction, and separate the labeled oligonucleotide from the excess [γ-^{32}P]-ATP by spun-column chromatography. (The author uses a 1-mL syringe plugged with polyallomer wool and filled with Sephadex G-50M. Spin at 1000–2000*g* in a bench-top centrifuge with the syringe supported in a 15-mL Falcon tube.)
4. The labeling reaction should yield 1–2 × 10^8 cpm in total Check the incorporation of radioisotope by counting 1 µL of the spun-column eluate by liquid scintillation counting.
5 To the labeled oligonucleotide (approx 100 µL) add. 11 µL 10X T4 DNA ligase buffer, 2 µL 10 m*M* ATP, 2 µL (20 U) T4 DNA ligase.
6 Ligate overnight at 15–18°C

3.4. Screening and Selection of Positive Plaques

Before embarking on library screening, it is advisable to determine the optimum conditions for binding in terms of specific probe sequence, nonspecific competitor DNA, and binding buffer composition. These points are considered in Notes 4–7.

1. For 10–20 filters, place 500 mL of South-western block in a tray or large sandwich box Immerse the filters from Section 3.2. in the blocking solution one at a time, ensuring that both surfaces of each filter come into contact with the solution.
2 Block overnight at 4°C with gentle shaking

3. Make up the probe mixture by adding the concatenated probe at 5×10^5–1×10^6 cpm/mL to 100 mL of TNE-50 together with nonspecific competitor DNA (Poly[dA]/Poly[dT]) at 10 μg/mL (*see* Note 6).
4. Probing is most conveniently carried out in 15-cm Petri dishes containing 50 mL of probe mix each. Place the probe solution into each of two 150-mm plastic Petri dishes, and lay the filters one at a time in the probe mixture, protein side up, placing only five to six filters in each dish.
5. Incubate for 1 h at room temperature (*see* Note 7), using a slowly moving orbital platform shaker to keep the filters floating freely. Filters can be processed in batches, reusing the probe solution and keeping the filters awaiting probing in blocking solution.
6 Remove the probed filters to a large tray or lunch box, and wash three to four times for 5–10 min each time in 200–300 mL of TNE-50 at room temperature, making sure that the filters remain free in the solution and do not stick together. Washing can be performed on an orbital platform shaker with fairly vigorous movement
7. Blot the filters dry, and expose to X-ray film, including fluorescent or radioactive markers to facilitate orientation of the filters to the developed film (*see* Notes 8 and 9).
8. The probe mixture may be retained and kept at –20°C for up to 1 wk, and may be used for second-round screens after adding fresh DTT immediately prior to use.

3.5. Guanidinium Chloride (GuHCl) Denaturation/Renaturation

Some DNA-binding proteins may be inactive as bacterially produced fusion proteins because of insolubility or incorrect protein folding. DNA-binding activity can sometimes be recovered by denaturation in GuHCl followed by slow renaturation. This procedure is preferably applied immediately after making the filter lifts, or if initial screening by the standard method has been unfruitful, the filter-bound proteins may be denatured as follows and then reprobed.

1. Immerse the filters one at a time in 6*M* GuHCl using a small tray or sandwich box containing 40 mL of solution/15-cm filter.
2. Agitate on a slowly moving orbital shaker for 10 min at room temperature.
3. Remove the GuHCl solution, discard one-half of it, and make up the volume with TNE-50. Mix and add back to the filters. Shake for a further 10 min.
4. Repeat this dilution of the GuHCl every 10 min seven more times, and finally discard the solution and replace with TNE-50. Wash for a further 10 min.
5. Block the filters overnight in South-western block.
6 Probe as described in Section 3.4.

3.6. Plaque Purification

Once positives are detected on duplicate filters (*see* Note 9), corresponding plugs of agar should be picked from the plate for further rounds of screening and for phage purification.

1 Using the orientation holes in the agar, align the plate with the developed autoradiograph, and use the wide end of a Pasteur pipet to pierce the agar.
2. Transfer the resulting agar plug into 1 mL of SM buffer containing a drop of chloroform in a glass bijou bottle.
3 Shake the bottles gently to speed phage elution (2 h to overnight).
4. Plate out the titered, eluted phage, but this time at a density of 500–1000 plaques on a 9-cm plate. Duplicate lifts are not necessary for second and further screens.
5 Screen the second-round filters, and pick positive areas of agar using the narrow end of a Pasteur pipet, again into 1 mL of SM plus a drop of chloroform.
6. Replate the phage eluted from these plugs at lower density on 9-cm plates for third-round screens.
7 Pick single-well isolated plaques if possible.
8 Replate at about 50 plaques/plate. All plaques should be positive on screening If not, continue with another round of plaque purification.

4. Notes

1. The protocols given here are written assuming that the reader will be using a λgt11 cDNA library *(2)* However, the method can be used with λ ZAP libraries with only minor modification, specifically, the use of *E coli* XL1-Blue rather than Y1090 for plating cells and the growth of the phage at 37°C NZY agar is also recommended for λ ZAP growth, but the author has found LB agar supplemented with 10 m*M* $MgSO_4$ to be satisfactory. Suitable cDNA libraries can be constructed by the investigator, can be obtained commercially, or may be available from other laboratories. The library should originate from a cell line or tissue known to express the DNA-binding activity of interest at a reasonable level, and for vertebrate organisms, should have a complexity of at least 10^6 independent recombinants and preferably in excess of 5×10^6 Remember that unless a directional cloning method has been employed during the library's construction, only one in six inserts will be in the correct orientation and frame to generate a β-galactosidase fusion protein. However, the author has found that in practice, proteins are often translated from an internal AUG codon. Although it is not necessary for clones to be full-length, sufficient coding information to generate a complete DNA-binding domain and possibly a dimerization domain is essential. Generally speaking, mixed oligo-dT primed and random primed libraries probably give the best results in this kind of exercise. If it is known that the DNA-binding activity resides in a particularly large polypeptide, then random priming will increase the probability of encountering clones encoding the DNA-binding activity of interest. Notably, however, smaller proteins may also be difficult to clone from oligo dT-primed libraries if they possess particularly long 3'-untranslated regions.
2. South-western blotting is a variation of the traditional Western blotting technique. Briefly, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer onto a nitrocellulose membrane, electrophoresed DNA-binding proteins are detected with a labeled DNA probe (rather than by immunological means). The probe can be prepared in exactly the same way as described in Sec-

tion 3.3., and the filter treated identically to the filter lifts in Sections 3.4. and 3.5. The method therefore embodies the technique used to screen the library. The sample material could be whole-cell extract, nuclear extract, or partially purified DNA-binding protein.

3. The author has found a density of 5×10^4 PFU/150-mm plate to be optimum.
4. The nature of the binding site probe is critical for successful cDNA library screening. In general, the binding site is chosen on the basis of gel retardation or other experiments, and should be bound specifically by the factor of interest. Probes containing multiple binding sites give good signals and may take the form of a single, long oligonucleotide containing several sites for the factor of interest (*see* Hai et al. *[4]* for example), a restriction fragment containing multiple cloned binding site oligonucleotides or as described in this chapter, a concatenated binding site-containing oligonucleotide *(6)*. For the construction of concatenated probes, the specific binding sequence for the factor of interest should be contained in a 20- to 25-mer double-stranded oligonucleotide with free unphosphorylated 5'-ends to facilitate labeling with [γ-^{32}P]-ATP and compatible "sticky ends" to allow ligation. A consideration when designing a probe is to avoid generating binding sites for other factors at the ligated junctions of the a concatenated probe
5. The author found TNE-50 to be a suitable buffer for probe binding and filter washing. It is a low-salt (50 m*M* NaCl) buffer selected to encourage the binding of bacterially synthesized proteins, which may bind DNA relatively poorly owing to such factors as lack of posttranslational modification. Binding activity in TNE-50 should preferably be tested in advance for the factor of interest by either gel retardation or South-western blot analysis.
6. Gel retardation analysis can also be used to determine the best nonspecific competitor DNA to use. Poly (dA)/poly (dT) was suitable, but poly (dI)/poly (dC) or herring sperm DNA may be more suitable for some factors.
7. The described hybridization conditions are given as a guide. Longer incubation times possibly at a lower temperature may improve the signal from a poorly binding factor. Similarly, the washing conditions described are the ones previously found to be the most effective. Longer washes at room temperature may result in loss of signal, but longer washes at lower temperatures may reduce background without significant loss of bound probe.
8. Using Kodak X-omat AR film and an intensifying screen, an overnight exposure at –70°C should be sufficient to detect clones giving strong signals. However, if the background allows, a second, longer exposure (1 wk) is also recommended in order to detect clones giving weaker signals.
9. Genuine positive plaques will give a signal from both of the duplicate filters, although the signal may be stronger on one duplicate than on the other.

References

1. Singh, H., LeBowitz, J. H., Baldwin, A. S., and Sharp, P. A. (1988) Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. *Cell* **52,** 415–423.

2. Huynh, T. V., Young, R. W., and Davis, R. W. (1988) Construction and screening of cDNA libraries in lambda gt11 and lambda gt10, in *Cloning: A Practical Approach*, vol. 1 (Glover, D. M., ed.), IRL, Oxford, UK, pp. 49–78
3 Maekawa, T., Sakura, H., Kanei Ishii, C , Sudo, T., Yoshimura, T , Fujisawa, J., Yoshida, M., and Ishii, S. (1989) Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J* **8,** 2023–2028.
4. Hai, T , Liu, F., Coukos, W. J., and Green, M R. (1989) Transcription factor ATF cDNA clones· an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev* **3,** 2083–2090.
5 Poli, V., Mancini, F. P., and Cortese, R (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63,** 643–653.
6 Cowell, I. G., Skinner, A., and Hurst, H. C. (1992) Transcriptional repression by a novel member of the bzip family of transcription factors. *Mol. Cell Biol* **12,** 3070–3077.
7 Kageyama, R and Pastan, I. (1989) Molecular cloning and characterisation of a human DNA binding factor that represses transcription. *Cell* **59,** 815–825
8. Williams, T. M., Moolten, D., Burlein, J , Romano, J , Bhaerman, R , Godillot, A., Mellon, M , Rauscher, F. J., III, and Kant, J. A. (1991) Identification of a zinc finger protein that inhibits IL-2 expression *Science* **254,** 1791–1793.