Purification of Sequence-Specific DNA-Binding Proteins by Affinity Chromatography

Many biological processes, such as recombination, replication, and transcription, involve the action of sequence-specific DNA-binding proteins. Analysis and purification of these proteins by conventional chromatographic methods is often difficult because DNA-binding proteins typically make up <0.01% of the total cellular protein. Various methods have been developed for purifying such proteins. Affinity chromatography is a very effective means of purifying a protein based on its sequence-specific DNA-binding properties and is relatively straightforward if proper care is taken. The affinity chromatography procedure described in this unit uses DNA containing specific recognition sites for the desired protein that has been covalently linked to a solid support. Researchers in the past have purified proteins based on the proteins' ability to bind DNA, only to be disappointed to find that the DNA-binding activity of their highly purified products was not sequence-specific. This situation can be avoided by carefully performing all of the preliminary steps and experimental controls.

Basic Protocol 1 describes preparation of a DNA affinity resin, including cyanogen bromide (CNBr) activation of the agarose support. The Alternate Protocol provides a method to couple DNA to commercially available CNBr-activated Sepharose. Support Protocol 1 describes how to purify crude synthetic oligonucleotides by gel electrophoresis prior to preparation of the affinity resin. Basic Protocol 2 outlines the affinity chromatography procedure. Support Protocol 2 describes determination of the appropriate type and quantity of nonspecific competitor DNA that should be used in the procedure and its preparation. Parameters essential to the success of an affinity chromatography experiment are discussed in detail in the Commentary. Figure 9.6.1 provides a summary diagram of the entire chromatography procedure.

PREPARATION OF DNA AFFINITY RESIN

BASIC PROTOCOL 1

Correct choice of oligonucleotide sequence (discussed in detail in the Commentary) and preparation of the affinity resin are probably the most important parts of the affinity chromatography procedure. Preparation of affinity resin can be broken down into four steps: (1) preparing oligonucleotides; (2) activating Sepharose; (3) coupling DNA to resin; and (4) blocking unreacted CNBr. The first step requires highly purified oligonucleotides that contain the recognition sequence for the desired protein. Once purified, the complementary oligonucleotides are annealed, phosphorylated with T4 polynucleotide kinase, and ligated into long, multimeric chains (averaging ≥10-mers) with T4 DNA ligase. Sepharose CL-2B is activated with CNBr, the ligated DNA added, and the coupling reaction carried out overnight. Because CNBr is very toxic, researchers may prefer to employ the Alternate Protocol, which uses commercially available CNBr-activated Sepharose and therefore avoids direct handling of CNBr. After the coupling reaction, the remaining reactive groups are blocked with ethanolamine. This protocol is designed to prepare 10 ml of affinity resin.

NOTE: Glass-distilled or other high-quality water should be used throughout these procedures.

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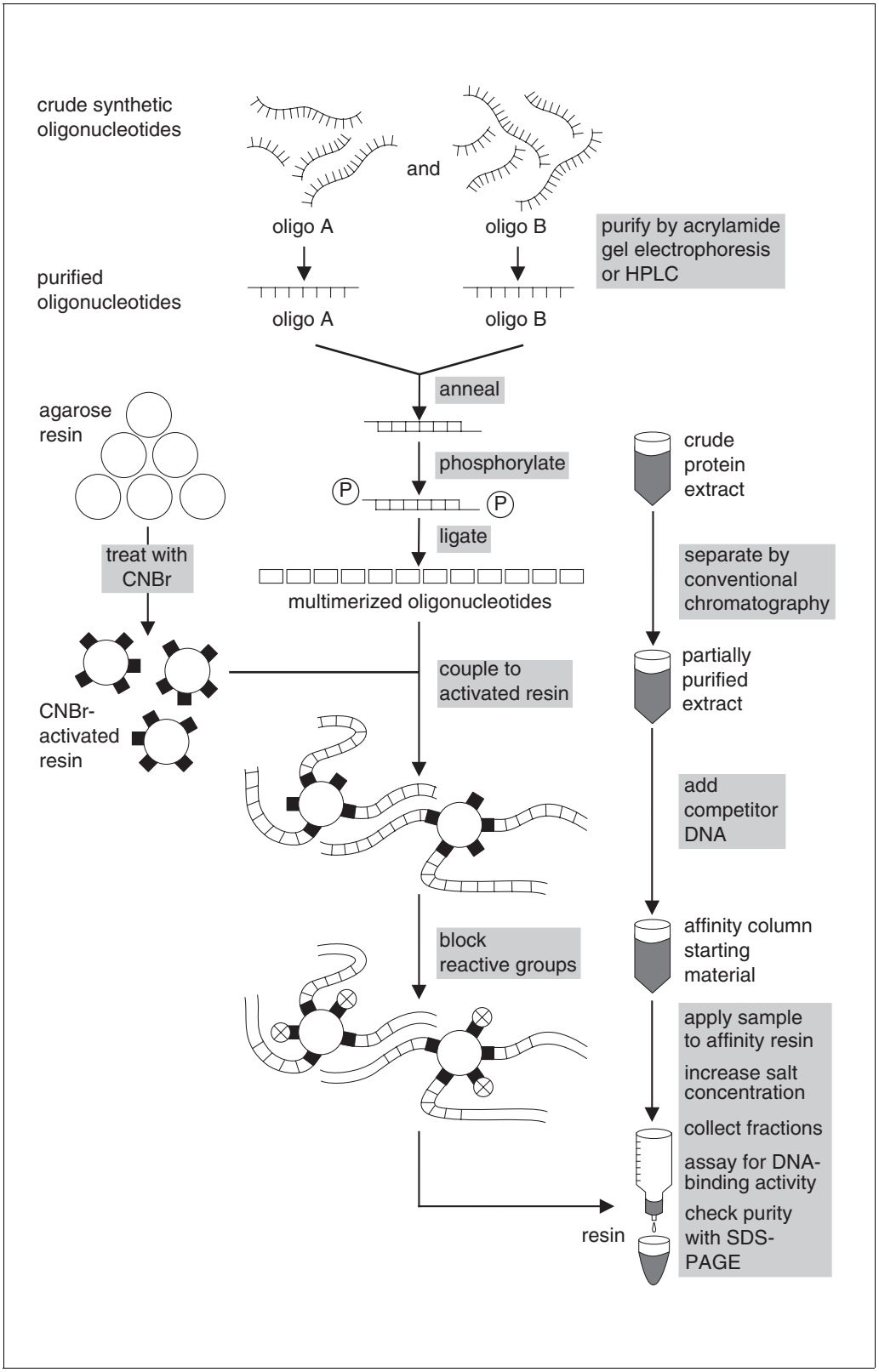

Figure 9.6.1 Purification of sequence-specific DNA-binding proteins with DNA affinity chromatography. Shown are the steps required to perform an affinity chromatography experiment using the methods described in this unit.

Materials

440 µg each of two synthetic oligonucleotides with desired binding site (see Support Protocol 1; or commercial HPLC-purified)
TE buffer, pH 7.8 (*APPENDIX 2E*)
10× T4 polynucleotide kinase buffer (see recipe)
20 mM ATP (Na^+ salt), pH 7.0
150 mCi/ml [γ-^{32}P]ATP (6000 Ci/mmol)
10 U/µl T4 polynucleotide kinase (New England Biolabs)
10 M ammonium acetate (*APPENDIX 2E*)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
24:1 (v/v) chloroform/isoamyl alcohol
3 M sodium acetate (*APPENDIX 2E*)
100% and 75% ethanol
10× linker/kinase buffer (see recipe)
6000 U/ml T4 DNA ligase (measured in Weiss units; New England Biolabs)
Buffered phenol
Isopropanol (2-propanol)
Sepharose CL-2B (Pharmacia Biotech)
Cyanogen bromide (CNBr; Aldrich)
N,*N*-dimethylformamide
5 N NaOH (*APPENDIX 2E*)
10 mM and 1 M potassium phosphate buffer, pH 8.0 (*APPENDIX 2E*)
1 M ethanolamine hydrochloride, pH 8.0 (see recipe)
NaOH, solid
Glycine
1 M KCl (*APPENDIX 2E*)
Column storage buffer (see recipe)

15-ml screw-cap polypropylene tubes
Heating blocks or water baths, 15°C, 37°C, 65°C, and 88°C
60-ml coarse-sintered glass funnel
Rotating wheel

5′-phosphorylate the oligonucleotides

1. In a 1.5-ml microcentrifuge tube, prepare a mixture containing 440 µg of each oligonucleotide in TE buffer in a total volume of 130 µl. Add 20 µl of 10× T4 polynucleotide kinase buffer. Incubate 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and 5 min at room temperature.

   A 1-µmol synthesis of oligonucleotide should yield enough purified DNA for ~20 ml of affinity resin.

2. Divide mixture in half in separate microcentrifuge tubes. To each 75-µl aliquot, add 15 µl of 20 mM ATP (pH 7.0), ~5 µCi [γ-^{32}P]ATP, and 10 µl of 10 U/µl T4 polynucleotide kinase (100 U total). Incubate 2 hr at 37°C.

   Dividing the reaction in half at this point facilitates the steps that follow. To add the labeled ATP, do not thaw, but simply touch (do not jab) the top of the frozen [γ-^{32}P]ATP with a yellow pipet tip and transfer the resulting tiny amount to the reaction tube.

3. Inactivate the kinase by adding 50 µl of 10 M ammonium acetate and 100 µl water to each tube and heating 15 min at 65°C. Allow to cool to room temperature.

Purify phosphorylated oligonucleotides

4. Add 750 µl of 100% ethanol and mix by inversion. Microcentrifuge at high speed 15 min at room temperature to pellet DNA. Discard supernatant.
5. Resuspend each pellet in 225 µl TE buffer.
6. Add 250 µl of 25:24:1 phenol/chloroform/isoamyl alcohol to each tube. Vortex 1 min. Microcentrifuge 5 min at high speed to separate phases. Transfer the aqueous phase (upper layer) to a new tube.
7. Add 250 µl of 24:1 chloroform/isoamyl alcohol to aqueous phase. Vortex 1 min. Microcentrifuge 5 min at high speed to separate phases. Transfer the aqueous phase (upper layer) to a new tube.
8. Add 25 µl of 3 M sodium acetate to aqueous phase and mix by vortexing. Then add 750 µl of 100% ethanol and mix by inversion. Microcentrifuge 15 min at high speed to pellet DNA. Discard supernatant.
9. Wash pellet with 800 µl of 75% ethanol. Mix by vortexing. Microcentrifuge 5 min at high speed. Discard supernatant.
10. Dry pellet in vacuum evaporator (e.g., Speedvac).

Ligate oligonucleotides

11. Add 65 µl water and 10 µl of 10× linker/kinase buffer to each pellet. Dissolve DNA by vortexing. Add 20 µl of 20 mM ATP (pH 7.0) and 5 µl of 6000 U/ml T4 DNA ligase (30 Weiss units). Incubate ≥2 hr at room temperature or overnight at 15°C.

    Depending upon the oligonucleotides used, the optimal temperature for ligation will vary from 4° to 30°C. Short oligonucleotides (≤15-mers) tend to ligate better at lower temperatures (4° to 15°C), whereas oligonucleotides that have a moderate degree of palindromic symmetry tend to self-anneal and therefore ligate better at higher temperatures (15° to 30°C).

12. Monitor the ligation reaction by agarose gel electrophoresis, using 0.5 µl of ligation reaction per gel lane and including lanes containing size markers. Visualize DNA by ethidium bromide staining and UV photography.

    5′-phosphorylated oligonucleotides often do not ligate on the first attempt. If ligation has not occurred, extract the DNA once with 25:24:1 phenol/chloroform/isoamyl alcohol and once with 24:1 chloroform/isoamyl alcohol, then ethanol precipitate using sodium acetate as the precipitating salt. Dissolve the DNA in 225 µl TE, add 25 µl of 3 M sodium acetate, and reprecipitate with 750 µl of 100% ethanol. Wash with 75% ethanol, dry in Speedvac, and repeat ligation.

    The average length of the ligated oligonucleotides should be ≥10-mers. Ligating the oligonucleotides increases the binding capacity of the affinity resin and helps avoid potential problems due to steric hindrance between the factor and the Sepharose support. However, oligonucleotide multimers <10-mers will probably also work.

Purify oligonucleotide multimers

13. Add 100 µl buffered phenol to the 100-µl ligation reactions. Vortex 1 min. Microcentrifuge 5 min at high speed. Transfer aqueous phase (upper layer) to a new tube.
14. Add 100 µl of 24:1 chloroform/isoamyl alcohol to aqueous phase. Vortex 1 min. Microcentrifuge 5 min at high speed. Transfer aqueous phase (upper layer) to a new tube.
15. Add 33 µl of 10 M ammonium acetate to the aqueous phase. Mix by vortexing.

16. Add 133 µl isopropanol. Mix by inversion. Incubate 20 min at −20°C. Microcentrifuge 15 min at high speed to pellet DNA. Discard supernatant.

    The ammonium acetate/isopropanol precipitation removes residual ATP, which would otherwise interfere with coupling of the ligated DNA to the CNBr-activated Sepharose.

17. Add 225 µl TE buffer. Vortex to dissolve pellet. Add 25 µl of 3 M sodium acetate. Mix by vortexing. Add 750 µl of 100% ethanol. Mix by inversion. Microcentrifuge 15 min at high speed to pellet DNA. Discard supernatant.

18. Wash DNA twice with 75% ethanol. Dry pellet in vacuum evaporator.

19. Dissolve DNA in 50 µl water. Store at −20°C.

    Do not dissolve the DNA in TE buffer, as the Tris buffer in TE will interfere with the coupling reaction.

Prepare CNBr-activated Sepharose

It is best to assemble all equipment and reagents required for the activation and coupling reactions *before* proceeding with the following steps.

20. Place 10 to 15 ml (settled bed volume) of Sepharose CL-2B in a 60-ml coarse-sintered glass funnel and wash extensively with 500 ml water.

    To wash, add water to resin in funnel, stir gently with a glass rod, and remove water by vacuum suction, making sure to release vacuum before the resin is suctioned into a dry cake. Repeat until all 500 ml water is used.

21. Transfer moist Sepharose resin to a 25-ml graduated cylinder, estimating 10 ml of resin. Add water to 20 ml final volume. Transfer the resulting slurry to a 150-ml glass beaker containing a magnetic stir bar. Place beaker in a water bath equilibrated to 15°C and set up over a magnetic stirrer in a fume hood. Turn on stirrer to slow medium speed.

    Keep the water bath at 15°C by periodically adding small chunks of ice as needed. Occasional variation of a degree or two is not critical.

22. In the fume hood, measure 1.1 g CNBr into a 25-ml Erlenmeyer flask, keeping the mouth of the flask covered with Parafilm or a ground glass stopper as much as possible (it is better to have slightly more than 1.1 g than slightly less). Add 2 ml *N,N*-dimethylformamide (the CNBr will dissolve instantly). Over the course of 1 min, add the resulting CNBr solution dropwise to the stirring Sepharose slurry.

    CAUTION: *CNBr is highly toxic and volatile. Use only in a fume hood with extreme caution. Observe appropriate decontamination and disposal procedures.*

23. Immediately add 5 N NaOH as follows: add 30 µl to the stirring mixture every 10 sec for 10 min until 1.8 ml NaOH has been added.

    It is convenient to measure 1.8 ml NaOH into a small tube before addition to the reaction to avoid having to change pipet tips during the 10-sec additions.

24. Immediately add 100 ml ice-cold water to the beaker and pour the mixture into a 60-ml coarse-sintered glass funnel.

    IMPORTANT NOTE: *At this point, it is very important to avoid suction-filtering the resin into a dry cake. If the resin is accidentally dried into a cake, do not use; instead, repeat steps 20 to 24 with fresh resin.*

25. Still working in the fume hood, wash the resin in the funnel with four 100-ml washes of ice-cold (≤4°C) water followed by two 100-ml washes of ice-cold 10 mM potassium phosphate, pH 8.0.

26. Immediately transfer the resin to a 15-ml polypropylene screw-cap tube and add ~4 ml of 10 mM potassium phosphate (pH 8.0) until the resin has the consistency of a thick slurry.

Couple oligonucleotide multimers to CNBr-Sepharose

27. Immediately add the two 50-µl aliquots of DNA from step 19. Incubate on a rotating wheel overnight (≥8 hr) at room temperature.

28. In the fume hood, transfer the resin to a 60-ml coarse-sintered glass funnel and wash with two 100-ml washes of water and one 100-ml wash of 1 M ethanolamine hydrochloride, pH 8.0.

    Using a Geiger counter, compare the level of radioactivity in the first few milliliters of filtrate with the level of radioactivity in the washed resin to estimate the efficiency of incorporation of DNA to the resin. Usually, all detectable radioactivity is present in the resin.

29. In the fume hood, transfer the resin to a 15-ml polypropylene screw-cap tube and add 1 M ethanolamine hydrochloride (pH 8.0) until the mixture is a smooth slurry. Incubate the tube on a rotating wheel 2 to 4 hr at room temperature.

    This step inactivates unreacted CNBr-activated Sepharose.

    It is important to clean up all CNBr waste carefully. In the fume hood, add solid NaOH and glycine (~10 to 20 mg/ml) to inactivate the CNBr. Soak contaminated instruments in a similar solution. Let sit overnight in the fume hood, then discard.

30. Wash the resin in a 60-ml coarse-sintered glass funnel with 100 ml of 10 mM potassium phosphate (pH 8.0), 100 ml of 1 M potassium phosphate (pH 8.0), 100 ml of 1 M KCl, 100 ml water, and 100 ml column storage buffer.

31. Store the resin at 4°C (stable at least 1 year; do not freeze).

ALTERNATE PROTOCOL

COUPLING THE DNA TO COMMERCIALLY AVAILABLE CNBr-ACTIVATED SEPHAROSE

The major advantage of this alternate procedure is that it begins with commercially available CNBr-activated chromatography resin, avoiding the need for preparation of CNBr-activated resin (see Basic Protocol 1). However, commercial CNBr-Sepharose is considerably more expensive than the homemade variety; moreover, the resulting column tends to run more slowly than one prepared as described above. Both resins are effective, leaving it up to the researcher's discretion which to use.

Additional Materials *(also see Basic Protocol 1)*

1 mM HCl (*APPENDIX 2E*), prepared fresh before use
CNBr-activated Sepharose 4B (Pharmacia Biotech)

1. Prepare oligonucleotide multimers by phosphorylation and ligation (see Basic Protocol 1, steps 1 to 19).

2. Weigh out 3 g CNBr-activated Sepharose 4B (1 g freeze-dried resin gives ~3.5 ml final gel volume).

3. Place the dry resin in a 15-ml conical polypropylene tube. Hydrate resin with 10 ml of 1 mM HCl and mix gently by flicking and inverting the tube. After 1 min, transfer slurry to a 60-ml coarse-sintered glass funnel. Wash and swell the beads by gradually pouring 500 ml of 1 mM HCl through the funnel (this will take ~15 min).

4. Wash the resin with 100 ml water and then with 100 ml of 10 mM potassium phosphate, pH 8.0.
5. Proceed with coupling of oligonucleotide multimers to the resin (see Basic Protocol 1, steps 26 to 31).

PURIFICATION OF OLIGONUCLEOTIDES BY PREPARATIVE GEL ELECTROPHORESIS

SUPPORT PROTOCOL 1

Preparation of affinity resin requires a large amount (1-µmol synthesis) of purified synthetic oligonucleotides. The oligonucleotides used to prepare the affinity resin must be of high purity because contaminating, incompletely synthesized oligonucleotides can interfere with the ligation reaction. HPLC-purified oligonucleotides are of sufficient purity for preparation of DNA affinity resins; however, it is normally expensive to obtain such oligonucleotides. An alternative is to purify crude oligonucleotides by the following method.

Additional Materials *(also see Basic Protocol 1)*

16% polyacrylamide-urea gel (see recipe)
Oligonucleotides to be purified
Formamide loading buffer (see recipe)
sec-butanol (2-butanol)
Diethyl ether
1 M $MgCl_2$ (*APPENDIX 2E*)

Saran wrap or other UV-transparent plastic wrap
Intensifying screen (e.g., Lightning Plus, NEN Life Sciences)
Hand-held short-wavelength UV light source
Silanized glass wool
Dry ice/ethanol bath (−78°C)

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*UNIT 10.3*)

Purify oligonucleotides by gel electrophoresis

1. Prepare a 20 cm × 40 cm × 1.5 mm denaturing polyacrylamide gel with four wells 3 cm in width, using 16% polyacrylamide/urea for separating oligonucleotides ~10 to ~45 bases long (or 8% or 6% polyacrylamide/urea for longer oligonucleotides). Let gel polymerize for ≥30 min, then prerun at 30 W for ≥1 hr.
2. Dissolve each oligonucleotide in formamide loading buffer to 200 µl final. Heat 15 min at 65°C to remove any secondary structure in the DNA. Load 50 µl of samples into separate wells, and run the gel at 30 W for ~4 hr.

   The amount of oligonucleotide that is prepared in a 1-µmol synthesis (~1 to 2 mg) can be loaded on one gel (0.25 µmol per 3-cm well). This is the maximum amount that can be applied to the gel without overloading it.

   It takes ~4 hr for the bromphenol blue in the loading buffet to migrate three-quarters of the way down the gel, which is far enough to purify oligonucleotides of 15 to 30 bases. In a 16% gel, bromphenol blue comigrates with ~10-base oligonucleotides and xylene cyanol comigrates with ~30-base oligonucleotides. If the oligonucleotide is 25 to 35 bases long, it is recommended that the formamide loading buffer be made without xylene cyanol.

Visualize gel by UV shadowing

3. Remove one of the glass gel plates and cover the gel with Saran wrap. Flip gel over and remove the other plate so that the gel is lying on the Saran wrap. Cover the other side of the gel with Saran wrap.

4. In a darkroom, lay the gel on an intensifying screen and hold a hand-held short-wavelength UV light source directly over it to visualize the DNA. Identify the major oligonucleotide band and mark its position directly on the Saran wrap using a marker, making sure that the light is directly over the band.

   The major band (usually the largest oligonucleotide but sometimes the next-to-largest) should be visible as a thick, dark band in the gel.

Purify oligonucleotides from gel

5. Carefully cut out the band with a razor blade, trying to avoid shredding the gel material or pulverizing the gel slice into small pieces.
6. Soak the gel piece in 5 ml TE buffer in a 15-ml polypropylene tube overnight at 37°C with shaking.
7. Place a silanized glass wool plug in a Pasteur pipet and prerinse with ~5 ml water. Filter the supernatant containing the DNA through the glass wool.
8. Concentrate the DNA to ≤180 µl by repeated extractions with *sec*-butanol.
9. Extract the DNA once with diethyl ether and place in a vacuum evaporator (e.g., Speedvac) until all traces of ether are removed.
10. Adjust the volume of the liquid to 180 µl with TE buffer. Add 20 µl of 3 M sodium acetate and 2 µl of 1 M $MgCl_2$. Mix by vortexing.
11. Add 600 µl of 100% ethanol. Mix by inversion. Chill 10 min in dry ice/ethanol. Let stand 5 min at room temperature. Microcentrifuge 15 min at high speed, room temperature, to pellet DNA. Discard supernatant.
12. Add 180 µl TE buffer. Dissolve pellet by vortexing. Add 20 µl of 3 M sodium acetate and 2 µl of 1 M $MgCl_2$. Mix by vortexing.
13. Add 600 µl of 100% ethanol. Mix by inversion. Chill 10 min in dry ice/ethanol. Let stand 5 min at room temperature. Microcentrifuge 15 min at high speed, room temperature. Discard supernatant.
14. Add 800 µl of 75% ethanol. Mix by vortexing. Microcentrifuge 5 min at high speed, room temperature. Discard supernatant.
15. Carefully dry pellet in vacuum evaporator (sometimes static electricity will cause the pellet to jump out of the tube).
16. Dissolve DNA in 200 µl TE buffer and measure A_{260} and A_{280}. Store at −20°C indefinitely.

    For sequence-specific DNA affinity resins, assume that 1 A_{260} unit = 40 µg/ml DNA.

BASIC PROTOCOL 2

DNA AFFINITY CHROMATOGRAPHY

Affinity chromatography is performed by combining a partially purified protein sample with appropriate competitor DNA, pelleting the insoluble protein-DNA complexes by centrifugation, and loading the resulting soluble material by gravity flow onto the affinity resin. Nonspecific DNA-binding proteins flow through the column while the specific protein is retained by the column. The protein is then eluted by gradually increasing the salt concentration of the buffer and individual fractions are tested for DNA-binding activity and purity. Fractions containing appropriate DNA-binding activity can be reapplied to the affinity resin if further purification is desired. By using two sequential affinity chromatography steps, a typical protein can be purified 500- to 1000-fold with ~30% yield. The method described below is performed with buffer Z, but many other buffers will work as well. Buffer choice is addressed in the Commentary.

Materials

Prepared DNA affinity resin (see Basic Protocol 1 or Alternate Protocol)
Buffer Z or other column buffer (e.g., buffers Z^e or TM; see recipes) made with varying KCl concentrations (buffers Z/0.1 M KCl through Z/1 M KCl)
Partially purified protein fraction dialyzed against buffer Z/0.1 M KCl
Nonspecific competitor DNA (see Support Protocol 2)
Column regeneration buffer (see recipe)
Column storage buffer (see recipe)

Disposable chromatography column (Poly-Prep, Bio-Rad)
Sorvall SS-34 rotor or equivalent
Liquid nitrogen
Narrow glass rod, silanized

Additional reagents and equipment for DNA-binding assays (Brenowitz, et al., 1989; Buratowski and Chodosh, 1996; Baldwin et al., 1996), SDS-PAGE (*UNIT 10.1*), and silver staining (*UNIT 10.5*)

1. Equilibrate 1 ml settled bed volume of the DNA affinity resin in a disposable chromatography column with two 10-ml washes of buffer Z/0.1 M KCl.
2. Combine the partially purified protein fraction in buffer Z/0.1 M KCl with nonspecific competitor DNA as determined by DNA-binding studies (see Commentary and Support Protocol 2).
3. Incubate mixture 10 min on ice.
4. Centrifuge mixture 10 min at 12,000 × *g* (10,000 rpm in Sorvall SS-34 rotor), 4°C, to pellet insoluble protein-DNA complexes.
5. Load the supernatant onto the column at gravity flow (e.g., 15 ml/hr per column for Sepharose CL-2B).

   A single 1-ml column is sufficient for a standard nuclear extract (e.g., 12 liters of HeLa cells or 150 g of Drosophila embryos). When purifying a larger quantity of material, it is preferable to use multiple 1-ml columns. It is common practice to apply as much as 50 ml of protein sample onto a single 1-ml column.

   Typically, 1 ml of affinity resin contains ~80 to 90 μg DNA, which corresponds to a protein-binding capacity of 7 nmol/ml of resin, assuming one recognition site per 20 bp.

6. After loading the starting material, wash the column four times with 2-ml aliquots of buffer Z/0.1 M KCl, rinsing the sides of the column each time.

   It is very important to wash the affinity column thoroughly at this step by rinsing down the sides of the column with the 2-ml wash buffer aliquots; a single 8-ml wash is not effective.

   DNA affinity columns will often yield >100-fold purification of the desired factor. When a protein is purified 100-fold, however, a 1% contamination of the starting material due to inefficient washing of the column will lead to major contamination of the affinity-purified factor.

7. Elute the protein from the column by successive addition of 1-ml portions of buffer Z/0.2 M KCl, buffer Z/0.3 M KCl, buffer Z/0.4 M KCl, buffer Z/0.5 M KCl, buffer Z/0.6 M KCl, buffer Z/0.7 M KCl, buffer Z/0.8 M KCl, and buffer Z/0.9 M KCl, followed by three 1-ml aliquots of buffer Z/1 M KCl. Collect 1-ml fractions that correspond to the addition of the 1-ml portions of buffer. Quick-freeze the protein samples in liquid nitrogen and store at −80°C. Samples can generally be stored for at least 2 years.

It is convenient to save separate aliquots of each fraction for DNA-binding assays (20 µl each) and SDS-PAGE analysis (50 µl each).

8. Assay the protein fractions for the sequence-specific DNA-binding activity using a DNA-binding assay (Brenowitz, et al., 1989; Buratowski and Chodosh, 1996; Baldwin et al., 1996). Estimate the purity of the protein fractions by SDS-PAGE (*UNIT 10.1*) followed by silver staining to visualize the protein.

   If further purification is desired, combine the fractions that contain the activity and, depending on the KCl concentration, either dilute (using buffer Z without KCl) or dialyze (against buffer Z/0.1 M KCl) to 0.1 M KCl. Then combine the protein fraction with nonspecific competitor DNA (the amount and type of which must be determined experimentally as described in Support Protocol 2) and reapply to either fresh or regenerated DNA affinity resin.

   In some instances, proteins might still be bound to the affinity resin after the 1 M salt elution step (step 7). Thus, if the desired protein is not detected in either the column fractions or flowthrough, it is possible that the protein is still on the column, and a wash at a higher salt concentration (e.g., 2 M KCl) may be needed to elute the protein from the resin.

9. Regenerate the affinity resin as follows: At room temperature, stop the column flow and add 5 ml column regeneration buffer to the column. Stir the resin with a silanized narrow glass rod to mix the resin with the regeneration buffer. Let the buffer flow out of the column. Repeat step.

10. To store column, add 10 ml column storage buffer and allow to flow through. Repeat this wash, then close the bottom of the column and add another 5 ml buffer. Cover top of column and store at 4°C.

    The column may be stored for ≤1 year. Alternatively, the affinity resin may be removed from the column after the two washes and stored in a clean polypropylene tube with the 5 ml buffer.

SUPPORT PROTOCOL 2

SELECTION AND PREPARATION OF NONSPECIFIC COMPETITOR DNA

Proper use of competitor DNA is essential for successful purification of DNA-binding proteins by affinity chromatography. Common competitor DNAs include poly(dI-dC), poly(dA-dT), poly(dG-dC), and calf thymus DNA. The amount and type of competitor to use in a given experiment must be determined experimentally. A typical method is to mix a protein sample (the exact fraction that will be applied to the affinity resin) with varying amounts of different competitor DNAs and evaluate DNA binding using assays such as DNase I footprinting (Brenowitz et al., 1989) or gel mobility shifts (Buratowski and Chodosh, 1996). The competitor DNA that inhibits DNA binding the least should be used for DNA affinity chromatography. It is perhaps easiest to think of these binding experiments as scaled-down affinity columns. First, determine the highest amount of competitor DNA that can be added to a DNA-binding reaction that does not interfere with the binding of the sequence-specific factor. To move up to a full-scale experiment, use one-fifth of the amount that would be required if the binding reaction were directly scaled up.

The following example with a hypothetical factor M illustrates how to determine the amount of competitor to use in an affinity chromatography experiment. Using DNase I footprinting, a strong footprint was observed with 5 µl of a 0.4 M heparin fraction. Testing various competitors reveals that calf thymus DNA, poly(dI-dC), and poly(dG-dC) all strongly inhibit binding of factor M. However, no detectable inhibition of factor M binding is observed with 2 µg poly(dA-dT), weak but detectable inhibition is observed with 3 µg poly(dA-dT), and strong inhibition is observed with 4 µg poly(dA-dT). In this example,

with 5 µl of the 0.4 M heparin fraction, the highest amount of poly(dA-dT) that does not inhibit factor M binding is 2 µg. If 5 ml of the 0.4 M heparin fraction is available, the direct scale-up would be 1000-fold, so 1000 × 2 µg = 2000 µg poly(dA-dT) would be needed. Because in an affinity chromatography experiment one-fifth of the direct scale-up amount is used, the appropriate amount of poly(dA-dT) to add to 5 ml of the 0.4 M heparin fraction is 2000 µg × 1/5 = 400 µg.

The optimal amount of competitor DNA will vary with the purity of the partially purified protein sample. Fractions that contain more nonspecific DNA-binding proteins will normally require more competitor DNA and thus it is necessary to determine experimentally the optimal amount of DNA to use with each protein fraction. In addition, the amount of competitor DNA is estimated as the mass of DNA (in micrograms) to add per volume (in milliliters) of protein fraction; this is because it is likely that competitor DNA acts by forming a complex with high-affinity, nonspecific DNA-binding proteins in the crude extract. Different competitor DNAs can be used in a single experiment.

To prepare poly(dI-dC), poly(dG-dC), and poly(dA-dT), dissolve the desired amount of competitor DNA to a final concentration of 10 A_{260} units in TE buffer per 100 mM NaCl. Heat the sample to 90°C and slowly cool to room temperature over 30 to 60 min. If the average length of the DNA is >1 kb, degrade it by sonication. Estimate the length of the DNA by agarose gel electrophoresis.

REAGENTS AND SOLUTIONS

Buffer TM

50 mM Tris·Cl, pH 7.9 (*APPENDIX 2E*)
0 M *or* 1 M KCl
12.5 mM $MgCl_2$
1 mM dithiothreitol (DTT; add fresh just before use)
20% (v/v) glycerol
0.1% (v/v) Nonidet P-40 (NP-40)

Do not make a 10× buffer. To generate aliquots of buffer with the range of KCl concentrations described in the protocol, make two 500-ml batches of 1× buffer containing no KCl and 1 M KCl respectively, and mix together appropriate quantities. Store at 4°C. Just prior to use, place 1.5-ml aliquots of each concentration in separate 1.5-ml microcentrifuge tubes, and add DTT.

Buffer Z

25 mM HEPES (K^+ salt), pH 7.6
0 M *or* 1 M KCl
12.5 mM $MgCl_2$
1 mM DTT (add fresh just before use)
20% (v/v) glycerol
0.1% (v/v) NP-40
Adjust pH to 7.6 with KOH

Do not make a 10× buffer. To generate aliquots of buffer with the range of KCl concentrations described in the protocol, make two 500-ml batches of 1× buffer containing no KCl and 1 M KCl respectively, and mix together appropriate quantities. Store at 4°C. Just prior to use, place 1.5-ml aliquots of each concentration in separate 1.5-ml microcentrifuge tubes, and add DTT.

Buffer Z[e]

25 mM HEPES (K^+ salt), pH 7.6
0 M *or* 1 M KCl
1 mM DTT (add fresh just before use)
20% (v/v) glycerol
0.1% (v/v) NP-40
Adjust the pH to 7.6 with KOH

Do not make a 10× buffer. To generate aliquots of buffer with the range of KCl concentrations described in the protocol, make two 500-ml batches of 1× buffer containing no KCl and 1 M KCl respectively, and mix together appropriate quantities. Store at 4°C. Just prior to use, place 1.5-ml aliquots of each concentration in separate 1.5-ml microcentrifuge tubes, and add DTT.

Column regeneration buffer

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2E*)
1 mM EDTA, pH 8.0
2.5 M NaCl
1% (v/v) NP-40
Store at room temperature

The solution will be cloudy and separate into two phases (NP-40 and aqueous) upon storage. Mix by swirling and shaking just before use.

Column storage buffer

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2E*)
1 mM EDTA, pH 8.0
0.3 M NaCl
0.04% (w/v) sodium azide

Store at room temperature without sodium azide. Make a 4% (w/v) sodium azide stock solution and add just before use.

Ethanolamine hydrochloride, pH 8.0, 1 M

1 M ethanolamine
Adjust pH to 8.0 with HCl
Filter sterilize
Store at room temperature

Formamide loading buffer

90 ml deionized formamide
10 ml 10× TBE (see recipe below)
40 mg xylene cyanol
40 mg bromphenol blue
Store at −20°C

Linker-kinase buffer, 10×

660 mM Tris·Cl, pH 7.6 (*APPENDIX 2E*)
100 mM $MgCl_2$
100 mM DTT
10 mM spermidine
Store at −20°C
Add an extra 10 mM DTT just before use

16% polyacrylamide-urea gel

50 ml 40% (w/v) 19:1 acrylamide/bisacrylamide
12.5 ml 10× TBE (see recipe below)
62.5 g urea
17 ml H_2O
Mix, filter, briefly degas, and then add:
750 µl 10% (w/v) ammonium persulfate
20 µl TEMED

Pour immediately into prepared gel plates.

T4 polynucleotide kinase buffer, 10×

500 mM Tris·Cl, pH 7.6 (*APPENDIX 2E*)
100 mM $MgCl_2$
50 mM DTT
1 mM spermidine
1 mM EDTA, pH 8.0
Store at −20°C
Add an extra 50 mM DTT just before use

TBE (Tris/borate/EDTA) electrophoresis buffer, 10×

108 g Tris base (890 mM)
55 g boric acid (890 mM)
900 ml H_2O
40 ml 0.5 M EDTA, pH 8.0 (20 mM)
H_20 to 1 liter

COMMENTARY

Background Information

Purification of sequence-specific DNA-binding proteins has historically been a difficult task, mainly because such proteins are a small fraction of the total cellular protein. However, it is now possible to purify these factors quickly, simply, and effectively by using multimerized synthetic oligonucleotides that contain the recognition sequence for a particular DNA-binding protein. Early efforts with DNA-affinity chromatography involved adsorption or coupling of nonspecific DNA (such as calf thymus DNA) to either cellulose (Alberts and Herrick, 1971) or agarose (Arndt-Jovin et al., 1975) supports. These methods paved the way for the development of a variety of sequence-specific DNA affinity chromatography techniques, including the procedures described in this unit.

Other methods have been described to purify sequence-specific DNA-binding proteins, including chromatography using biotinylated DNA fragments attached to various supports by biotin-avidin or biotin-streptavidin coupling (*UNIT 10.6*; Chodosh et al., 1986; Kasher et al., 1986; Leblond-Francillard et al., 1987), oligonucleotides synthesized onto Teflon-based beads (Duncan and Cavalier, 1988), or synthetic oligonucleotide monomers attached to agarose supports (Wu et al., 1987; Blanks and McLaughlin, 1988; Hoey et al., 1993); and preparative gel mobility shifts (Gander et al., 1988). Although more than 50 sequence-specific DNA-binding proteins have been purified by the method described in this unit (Kadonaga, 1991, and references therein), it is likely that many of the techniques listed above are also effective for purifying sequence-specific DNA-binding proteins.

There are a variety of reasons that CNBr activation is commonly used in the preparation of affinity resins: it is simple, works well with agarose matrices, and is mild enough to bind ligands such as DNA. Briefly, the chemistry of the CNBr activation reaction is as follows. At high pH, the hydroxyl groups on the agarose resin react with CNBr. The majority of the CNBr added to the reaction reacts with water to yield inert cyanate ions, which is part of the reason such a large amount of CNBr is required. Additionally, the majority of the cyanate esters that are formed on the agarose either are hydrolyzed to form inert carbamate or react with the matrix hydroxyls to form imidocarbonates. The imidocarbonates that form can act effectively

as chemical cross-links, thus stabilizing the matrix (which is in most cases beneficial, particularly if the agarose resin chosen is not covalently cross-linked). The remaining active cyanate esters are coupled to the amino-containing ligands (in this case, oligonucleotides) at physiological pH. Finally, the unreacted cyanate esters are blocked with an excess of a suitable reagent, such as ethanolamine, to prevent coupling of the protein sample to the matrix (Janson and Rydén, 1989).

Critical Parameters and Troubleshooting

Basic strategy

The general approach to affinity purification of DNA-binding proteins is as follows. First, estimate the binding site of the desired protein by a technique such as DNase I footprinting (Brenowitz et al., 1989). If possible, it is best to survey a variety of promoters and enhancers. Next, determine the optimal conditions for protein binding to the DNA, considering factors such as temperature, ionic strength, pH, and Mg^{2+} concentration. Using conventional chromatography, partially purify the protein to remove contaminants such as nucleases that may degrade the affinity resin. Test a variety of nonspecific competitor DNAs, including poly(dI-dC), poly(dG-dC), poly(dA-dT), and calf thymus DNA to determine their effect on protein-DNA interactions. For best results, prepare two or more different DNA affinity resins with naturally occurring, high-affinity binding sites, preferably containing different flanking DNA sequences. The desired protein should bind with high affinity to both resins; if it does not, then it is likely that the protein that has been purified is not specific to the desired binding site. Finally, it is important to prepare a control resin that does not contain the recognition sequence for the desired protein. A control resin will make it possible to identify proteins that bind nonspecifically to DNA-Sepharose. Using this approach, it is possible to obtain a preparation containing a highly purified, sequence-specific DNA-binding protein.

Starting material and conventional chromatography

Extensive purification of the DNA-binding protein is not required for effective affinity chromatography. However, partial purification of the protein by conventional chromatography is recommended prior to affinity chromatography to remove proteases and nucleases that might degrade either the protein or the affinity resin. Keep in mind that sequence-specific and nonspecific DNA-binding proteins will often copurify in conventional chromatography, possibly because they may both interact with negatively charged polymers that resemble DNA. It is important to note that the most persistent contaminants in sequence-specific DNA-binding protein preparations are nonspecific, high-affinity DNA-binding proteins. Various conventional chromatographic methods may be employed prior to affinity purification of a DNA-binding protein; methods that have been used successfully in the past are discussed below.

Ion-exchange chromatography. One obvious first step might be cation-exchange chromatography with resins such as S-Sepharose Fast Flow/Mono S (Pharmacia Biotech), CM-52 (Whatman), CM Sepharose Fast Flow (Pharmacia Biotech), P11 phosphocellulose (Whatman), or Bio-Rex 70 (Bio-Rad). DNA-binding proteins (both specific and nonspecific) will normally bind to cation-exchange resins in buffers containing from 50 mM to 100 mM NaCl, and can be eluted with higher salt concentrations.

Affinity chromatography. Nonspecific DNA-cellulose (Alberts and Herrick, 1971) or DNA-agarose (Arndt-Jovin et al., 1975) resins can be used for a preliminary purification step. These resins are typically prepared with salmon sperm or calf thymus DNA. Also, sequence-specific DNA affinity resins can be prepared with oligonucleotides that do not contain binding sites for the desired factor. A strategy for chromatography with either nonspecific DNA affinity resin (e.g., see Rosenfeld and Kelly, 1986) or sequence-specific DNA affinity resins that lack the binding site for the desired factor (e.g., see Kaufman et al., 1989) is as follows. Once the protein sample is applied to a nonspecific DNA affinity resin, the desired factor will either (1) flow through the resin or (2) elute from the resin at a low salt concentration. High-affinity, nonspecific DNA-binding proteins should remain bound to the resin and will thus be separated from the desired protein. If this strategy is successful, it will not be necessary to add the competitor DNA prior to the subsequent sequence-specific affinity chromatography step because the high-affinity nonspecific DNA-binding proteins will already have been separated from the sample.

A resin that works well if the desired protein contains O-linked *N*-acetylglucosamine monosaccharide residues is wheat germ agglutinin-agarose. This method has been used successfully in the past with transcription factors such as Sp1 (Jackson and Tjian, 1989) and HNF1 (Lichtsteiner and Schibler, 1989).

Heparin-agarose chromatography. Heparin-agarose resins are also excellent for the purification of DNA-binding proteins because they possess properties similar to those of both ion-exchange and affinity resins. It is important to be aware that variability exists between different heparin-agarose preparations. This variability may be due to differences in the methods of coupling heparin to agarose, because alternate methods for coupling and blocking the agarose may produce different functional groups on the resin. If a particular batch of heparin-agarose is used successfully, it is wise to continue to use that batch to ensure reproducibility.

Gel-filtration chromatography. Finally, gel-filtration chromatography may be used effectively for partial purification of a DNA-binding protein because separation is based on size and shape, not DNA-binding properties. For example, gel filtration may be useful for separating a desired protein from contaminating nucleases and other nonspecific DNA-binding proteins, in contrast to chromatography using ion-exchange, heparin-agarose, and nonspecific DNA affinity resins that may actually enrich for DNA-binding proteins. In some cases gel filtration may be undesirable because significant sample dilution occurs; however, further concentration can be obtained by subsequent affinity chromatography.

DNA binding studies

Binding conditions. In order to use affinity chromatography to purify a DNA-binding protein, it is necessary to optimize binding conditions. It is important to determine these conditions experimentally, as ionic strength, temperature, pH, and presence or absence of Mg^{2+} can affect protein-DNA interactions. In addition, although HEPES and PIPES are often considered to be superior to Tris buffers, some commercially available preparations of HEPES and PIPES buffers contain contaminants that inhibit binding of proteins to DNA. Therefore, in some respects, it may be safer to use Tris buffer. It is also important to note that some factors, such as Sp1, bind with higher affinity to DNA in the absence of Mg^{2+} than in its presence. Thus, in some cases it may be useful to omit Mg^{2+} from the chromatography buffer. In addition, although the procedures in this unit use buffers containing KCl, NaCl could probably be substituted. Although it is usually preferable to handle proteins at 4°C, proteins have been described that will not bind DNA at 4°C, yet bind perfectly well at room temperature; in such cases the temperature at which the affinity chromatography is performed should be adjusted accordingly. Finally, to minimize nonspecific adsorption of affinity-purified proteins to plastic and glass, chromatography and storage buffers should contain a nonionic detergent such as NP-40 at a concentration of ~0.01% to 0.1% (v/v).

Binding assays. There are many methods for identifying proteins that are bound to DNA, including DNAse I footprinting (Galas and Schmitz, 1978; Brenowitz et al., 1989), Fe-EDTA footprinting (Tullius et al., 1987), methidiumpropyl-EDTA·Fe^{2+} (MPE; Hertzberg and Dervan, 1982), methylation interference (Baldwin et al., 1996), and gel mobility shift assays (Buratowski and Chodosh, 1996). Although binding information may be obtained from all these techniques, it is most straightforward to use DNase I footprinting to identify a protein binding site. Additionally, DNase I is active over a broad range of buffer conditions, which allows for considerable variation in binding conditions. Visualization of the protected region by footprinting makes designing oligonucleotides for affinity chromatography relatively simple (as discussed later in this section). Both DNase I footprinting and gel mobility shift assays are useful for assaying affinity-column fractions.

Design of oligonucleotides

Many different parameters are important in designing oligonucleotides for an affinity resin. The first step, as described above, is to determine the protein-binding site on the DNA. The DNA affinity resin should be prepared from naturally occurring, high-affinity binding sites. Using a consensus sequence may work in some instances, but in most situations a "real" binding site is best. Given that the length of the oligonucleotides can be reasonably flexible (14-mers to 61-mers have been used successfully), use of oligonucleotides that include the DNase I footprint as well as some flanking DNA (at least a few bases beyond the borders of the footprint) is recommended. In the early stages, it is better to copurify factors that bind

to the flanking region than to fail to purify the desired factor because the binding site is too short. It may be wise to avoid using oligonucleotides that are 21 or 42 bases in length because the DNA, if bent, might have a greater tendency to circularize during the ligation step. The oligonucleotides should also be designed with a single-stranded overhang, for two reasons: first, the ligation reaction will be more efficient (as compared to a blunt-end ligation), and second, it is likely that the DNA couples to the resin via the primary amine groups of the single-stranded overhang of the ligated multimers. The sequence GATC-XXX-BINDING SITE-XXX (where XXX represents the DNA flanking the binding site) works well, though it may also be convenient to use the sequences flanking the binding site as the overhang.

Inadvertent purification of nonspecific DNA-binding proteins

Throughout this unit, an attempt has been made to emphasize how to avoid inadvertent purification of nonspecific DNA-binding proteins. Unfortunately, this problem is quite common, particularly when the gel mobility shift assay is used to monitor DNA binding. Proper use of the control resin (to identify proteins that bind nonspecifically to DNA-Sepharose), two different affinity resins (to identify proteins that might be interacting with the linker DNA), and competitor DNA (to deplete nonspecific DNA-binding proteins from the extract) should virtually eliminate purification of nonspecific DNA-binding proteins. In addition, DNase I footprinting is recommended for assaying DNA binding because it allows visualization of the actual protein binding site and to confirm sequence specificity. To correlate DNA binding with a particular polypeptide, it would be informative to purify the polypeptide from a polyacrylamide gel by using the denaturation/renaturation procedure described by Hager and Burgess (1980). Two common nonspecific DNA-binding proteins that have been purified from HeLa cells by affinity chromatography are poly(ADP-ribose) polymerase, which has an M_r of 116,100 (Ueda and Hayaishi, 1985; Slattery et al., 1983), and the Ku antigen, which consists of two polypeptides of M_r 70,000 and 80,000 (Mimori et al., 1986).

Anticipated Results

After two rounds of affinity chromatography, it is possible to achieve a 500- to 1000-fold purification with a 30% overall yield. The concentration of affinity-purified proteins is typically 5 to 50 µg/ml. About 0.2 to 1 µl of protein (2 to 10 ng) can be expected to be sufficient for a "blanked-out" footprint (with ~10 fmol of DNA probe). Affinity resin is stable for >1 year if stored at 4°C. Affinity-purified proteins are typically stable for >2 years if stored at −80°C in an appropriate buffer and handled properly. Proteins should always be quick-frozen in liquid nitrogen and quick-thawed in either cold or room-temperature water (although if room-temperature water is used to accelerate thawing, the sample must not be allowed to warm up above 4°C). Although many DNA-binding proteins remain active after several freeze-thaw cycles, it is preferable to divide protein preparations into small aliquots rather than to freeze and thaw the entire sample many times.

Time Considerations

The time required to optimize DNA-binding conditions of a particular protein is variable and depends on assay type, probe preparation time, and competitor choices. The time to prepare extracts and to perform conventional chromatography is also variable depending on factors such as starting material and type of chromatography. Beginning with purified oligonucleotides, preparation of the DNA affinity resin takes 2 days (with either commercially available or homemade CNBr-activated resin). If the oligonucleotides are to be gel-purified, an additional day and a half should be added. Affinity chromatography takes ~4 to 8 hr, dependent mainly on the flow rate of the resin: Sepharose CL-2B runs at ~15 ml/hr, while Sepharose-4B runs somewhat slower. Therefore, it is possible to estimate chromatography time by calculating the volume of sample that is to be applied to the column plus 8 ml for the wash steps and 11 ml for the elution steps. If the volume of starting material is not too large, it is possible to assay the affinity column on the day it is run (either by DNA-binding assays, SDS-PAGE, or both). It is important to freeze the affinity fractions in liquid nitrogen and store at −80°C as soon as the column is complete.

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Key References

Kadonaga, J.T. 1991. See above.

Techniques paper, though less descriptive than this unit, containing a table that lists (with references) >50 sequence-specific proteins that have been purified using the affinity chromatography method described herein.

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First paper to describe affinity chromatography with multimerized oligonucleotides; details purification of transcription factor Sp1.

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