Published in final edited form as:

Methods Mol Biol. 2012 ; 786: 315–334. doi:10.1007/978-1-61779-292-2_19.

Proteomic methodologies to study transcription factor function

Harry W. Jarrett

Abstract

Transcription factors regulate transcription by binding to regulatory regions of genes including the promoter. Few of the transcription factors are well characterized and few promoters have been described in detail. New methods have been developed to improve both transcription factor and promoter characterization, some of which are discussed here. Trapping methodology applicable to both individual transcription factors and intact transcription complexes are described, as well as 2-dimensional gel electrophoresis, Southwestern blotting, and basic liquid chromatography/tandem mass spectrometry methodology. These methods have proved useful in the study of transcriptional regulation.

Keywords

Transcription; Regulation; DNA; Oligonucleotide; Promoter; Mass Spectrometry; Southwestern Blot

1. Introduction

Transcription factors are the activator and repressor proteins which bind to the promoter region of genes to regulate expression. One class of transcription factors is the specific transcription factors, which bind to response elements, individual DNA binding sites present throughout the promoter. Another group is often referred to as the general transcription factors, which for genes is generally the RNA polymerase II transcription (TFII) complex, which assemble a group of approximately 60 proteins and subunits near the initiation region where transcription will begin. Current models propose that the specific factors bind early and recruit the TFII complex, RNA polymerase II, and other proteins to initiate transcription (1).

In humans, there are approximately 23,000 genes (2), many of which have alternate promoters used at different stages of development or in different tissues. There are at least 1500 specific human transcription factors (3) (<http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home>). Very few of these promoters have ever been characterized in detail and only a few percent of the transcription factors are well-characterized. Thus, the task of characterizing the transcription factor proteome and clearly understanding genetic regulation is monumental. We have developed new methods, many of which are described here, which may be useful to scientists who also aim to comprehend transcriptional regulation. Here, we will focus on those techniques which may be unfamiliar to the reader and will not discuss reporter assays, chromatin immunoprecipitation (ChIP), promoter footprinting methodology, over-expression, or silencing methods which are also commonly used in transcription factor characterization.

Rather, we will focus on a basic set of techniques which can be used to purify and characterize proteins which bind to DNA. These include the isolation of nuclear extract from cultured cells, oligonucleotide purification and labeling, and the electrophoretic mobility shift assay for DNA-binding activity, which utilizes non-denaturing gel electrophoresis to detect the DNA-binding protein complexes. Methods for purifying individual transcription factors (oligonucleotide trapping) or complete transcription complexes (promoter trapping) are then presented. Further purification by two-dimensional gel electrophoresis and the use of Southwestern blotting techniques to detect specific, high affinity DNA-binding proteins are next presented. Finally, we will describe methods to digest the detected proteins with trypsin and their characterization by liquid chromatography-electrospray ionization-mass spectrometry.

2. Materials

2.1 Cell Culture and Nuclear Extract

1. Cell culture flasks of 182 cm² (Celltreat, Shirley, MA, USA).
2. Dulbecco's Modification of Eagle's medium (DMEM, Cellgro, Manassas, VA).
3. Heat inactivated adult bovine serum (Sigma Chemical Co., St. Louis, MO USA). The serum is inactivated by incubation at 56°C for 30 min and stored at 4°C prior to use.
4. PBS (10 x): 80 g NaCl, 2 g KCl, 27.2 g Na₂HPO₄·7H₂O, and 2.4 g KH₂PO₄ made up to 1 L with H₂O (Note 1). The PBS is autoclaved prior to use.
5. Trypsin-EDTA (1X) (Sigma).
6. 15 mL and 50 mL high density polyethylene, sterile, graduated culture tubes (Fisher Scientific, Pittsburgh, PA, USA).
7. Nuclear extract hypotonic buffer: 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, freshly added 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonylfluoride (PMSF).
8. Nuclear extract low-salt buffer: 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, freshly added 0.5 mM DTT and 0.2 mM PMSF.
9. Nuclear extract high-salt buffer: 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 1.6 M KCl, 0.2 mM EDTA, 25% glycerol, freshly added 0.5 mM DTT and 0.2 mM PMSF.
10. Nuclear extract dialysis buffer: 20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, freshly added 0.5 mM DTT and 0.2 mM PMSF.

2.2 Oligonucleotide Preparation (Note 2)

1. TE buffer: 10 mM Tris, 1 mM EDTA, pH 7.5. TE0.1 is TE containing 0.1 M NaCl. Similarly, TE0.4 and TE1.2 contain 0.4 and 1.2 M NaCl, respectively.
2. 0.5 M EDTA (free acid), titrated to pH 8 with 5 M NaOH.
3. 3 M sodium acetate solution: 40.8 g sodium acetate, 0.2 mL 0.5 M EDTA, 5 mL glacial acetic acid, and 66 mL water.

¹Water is >18 MΩ from a Millipore Synergy UV water purification unit.

²Oligonucleotides (1 μmol) are purchased from (Integrated DNA Technologies, Coralville, IA, USA). The company provides a molar absorptivity (E_{260nm}) used to calculate concentration.

2.3 Oligonucleotide Labeling

1. 10 $\mu\text{Ci}/\mu\text{L}$, 6000 Ci/mmol $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (PerkinElmer, Boston, MA), stored at -20°C .
2. T4 Polynucleotide kinase (10 units/ μL , New England BioLabs, Ipswich, MA, USA), stored at -20°C .
3. 1 mg/mL salmon sperm DNA (Sigma) in TE stored at -20°C .
4. 10% trichloroacetic acid (TCA): 10 g TCA dissolved to 100 mL in H_2O .
5. Supelco silanized glass wool (Sigma).
6. 17 $\times$ 100 mm sterile culture tubes (VWR, Sugar Land, TX, USA).
7. Bio-Gel P-6 fine resin (BioRad laboratories, Hercules, CA, USA). 10 g is suspended in 250 mL of TE in an autoclavable glass bottle and autoclaved for 45 min. After cooling and the resin has settled, excess liquid is removed to give a 1:1 slurry.

2.4 Electrophoretic Mobility Shift Assay (EMSA)

1. Acrylamide/Bis (29:1): dissolve 29 g acrylamide, 1 g N,N'-methylene-bis-acrylamide (Bis) to a final volume of 100 mL.
2. 5 X TBE: 30.03 g Tris base, 15.25 g boric acid, 10 mL 0.5 M EDTA, H_2O to 1,000 mL, autoclave 45 min, and store at room temperature.
3. 1 M Tris/HCl, pH 7.5: Dissolve 121.1 g Tris free base in 800 mL H_2O . Titrate to pH 7.5 with concentrated HCl. Adjust volume to 1 L and autoclave for 45 min.
4. 5 M NaCl. Dissolve 292.2 g NaCl to a total volume of 1 L with H_2O . Autoclave 45 min.
5. 5 X EMSA buffer (8 mL), 400 μL 1 M Tris-HCl (pH 7.5), 80 μL 0.5 M EDTA, 320 μL 5M NaCl, 1.6 mL glycerol, 3.2 μL 2-mercaptoethanol, and 5.6 mL H_2O . Stable for 1 week at room temperature.
6. Poly dI:dC (Sigma).
7. Bromophenol blue. 0.1 g bromophenol blue, 50 mL glycerol, adjust volume to 100 mL with water.

2.5 DNA-Sepharose Preparation

1. Cyanogen Bromide (CNBr)-Sepharose coupling buffer. 0.1 M NaHCO_3 , pH 8.3, 0.5 M NaCl.
2. CNBr-Sepharose blocking buffer. 0.1 M Tris, pH 8, 0.5 M NaCl.
3. 1.5 mL columns with polypropylene frits (Alltech).

2.6 Oligonucleotide Trapping Chromatography

1. EP24 (5'-GCTGCAGATTGCGCAATCTGCAGC-3').
2. ACEP24(GT)₅ (5'-GCTGCAGATTGCGCAATCTGCAGCGTGTGTGTGT-3'). The bold text highlights the binding site.
3. Heparin (Sigma, H-3393).
4. (dT)₁₈ (5'-TTTTTTTTTTTTTTTTTT-3').
5. Tween-20 (Fisher Chemical).

2.7 Promoter Trapping Chromatography

1. Taq polymerase (5 units/ μ L, New England BioLabs).
2. Lambda exonuclease (5 units/ μ L, New England BioLabs)).

2.8 Two-dimensional Gel Electrophoresis (2DGE)

1. 2DGE IEF rehydration buffer: 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.8% pH 3–10 Ampholytes (BioRad), 1% Zwittergent 3–10 (Sigma), 0.01% bromophenol blue.
2. 2DGE equilibration buffer: 50 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol, 0.001% bromophenol blue.
3. Blotting buffer: 10% methanol in 25 mM Tris, 192 mM glycine.
4. Blotting membrane is Sequi-blot PVDF membrane (0.2 μ m) (BioRad Laboratories). Prior to use, after cutting to size, the membrane is placed in methanol for 10 min to hydrate and then transferred to blotting buffer.

2.9 2DGE-Southwestern Blotting (2DGE-SW)

1. Southwestern Blotting binding buffer is 10 mM HEPES/NaOH, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 μ M $ZnSO_4$, and 0.1% Tween-20.

2.10 On-blot Trypsin Digestion for ESI-MS Analysis

1. SDS-stripping buffer: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol.

2.11 Capillary HPLC

1. Mobile phase A: 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA), from Fischer.
2. Mobile phase B: 90% acetonitrile (ACN)/0.5% HAc/0.005% TFA.

3. Methods

3.1 HEK293 Nuclear Extract

1. The method used is a minor modification of (4). Human Embryonic Kidney –293 (HEK293, American Type Culture Collection) cells are cultured in a NuAire IR Autoflow CO₂ water-jacketed incubator at 37° C with 5% CO₂ and 95% atmospheric air. The 182 cm² cell culture flasks are seeded with 5×10^6 HEK293 cells and grown in 60 mL/flask of DMEM containing 10% heat inactivated adult bovine serum. Cells are grown to over 90% confluence and eight flasks are harvested for a typical nuclear extract preparation.
2. Cells harvesting: the cells are first rinsed with PBS and harvested by adding 10 mL of trypsin-EDTA solution for 5 min at 37°C. The cells are then immediately suspended in 40 mL 4°C DMEM containing 10% inactivated adult bovine serum and removed from the flask.
3. All subsequent steps are on ice or at 4°C. The cells are centrifuged ($1,850 \times g$ for 5 min, 4°C) in 50 mL disposable, sterile plastic conical tubes, one tube per flask, and the cells are washed with 50 mL PBS. The cells are resuspended in sterile PBS, combined, and transferred to a 15 mL sterile, graduated conical tube and again

centrifuged. The final pellet will be approximately 2 mL and contain $2-3 \times 10^8$ cells. Carefully note the packed cell volume (pcv) and remove the supernatant.

4. Quickly resuspend the cells in 5 pcv of ice-cold nuclear extract hypotonic buffer, centrifuge the cells ($1,850 \times g$ for 5 min, 4°C), and discard the supernatant.
5. Resuspend the cells in 2 pcv of nuclear extract hypotonic buffer and allow to swell for 10 min on ice.
6. Transfer the cells to an ice-cold Dounce homogenizer and homogenize with the type B pestle using 10 slow up-and-down strokes.
7. Collect the nuclei by centrifugation ($3300 \times g$, 15 min) in a graduated conical tube. Carefully observe the packed nuclear volume (pnv). Discard the supernatant.
8. Add 0.5 pnv of nuclear extract low-salt buffer and transfer the nuclei to a clean, ice-cold Dounce homogenizer and homogenize with the type B pestle for two slow strokes.
9. While gently mixing, add drop-wise 0.5 pnv of nuclear extract high-salt buffer. The nuclei are then homogenized with two slow strokes with the pestle. Allow the nuclei to extract for 30 min on ice with gentle stirring. Transfer to a JA-20 centrifuge tube.
10. Pellet the extracted nuclei by centrifugation ($25,000 \times g$, 30 min). Save the supernatant and discard the pellet.
11. Dialyze the supernatant nuclear extract three times versus 50 volumes of nuclear extract dialysis buffer, allowing 4 h between each buffer change, 12 h total (Note 3).
12. Centrifuge the dialyzed nuclear extract ($25,000 \times g$, 20 min) and carefully remove the supernatant. Determine the protein concentration (5) and dilute as needed with fresh dialysis buffer to prepare 5 mg/mL protein. The yield is usually 1–2 mL. Prepare 50–100 μL aliquots and store frozen at -85°C . The nuclear extract can be stored for at least one year.

3.2 Oligonucleotide Cleanup

1. Oligonucleotides (Note 2) arrive dry after de-blocking in NH_4OH , which can interfere with labeling and coupling. They are ethanol precipitated.
2. Dissolve in 300 μL of TE buffer.
3. Add 30 μL of 3M NaOAc and 1mL of ethanol (absolute), mix, and allow to precipitate in the -85°C freezer for one hour (Note 4).
4. Centrifuge the tube in the cold room at $14,000 \times g$ for 12 min at 4°C . Carefully observe tube orientation so that the position of the pelleted DNA, which may not be visible, is known and can be avoided. Carefully remove the supernatant with a Pasteur pipette. Wash the pellet with 500 μL of ice-cold 70% ethanol by vortex mixing. Again centrifuge and carefully remove the supernatant. The tube is left open, covered with a tissue, and allowed to air-dry for 2 h at room temperature.

³Long dialysis is detrimental to activity. The dialysis procedure described gives high activity and thorough dialysis.

⁴For short oligonucleotides such as (AC)₅, increasing ethanol to 1.2 mL and using 75% ethanol for washing will increase yield.

5. If used for coupling to Sepharose, dissolve the pellet in water. For long term storage for other uses such as EMSA, dissolve the pellet in 500 μ L of TE. Allow 30 minutes, with occasional vortexing, for the pellet to dissolve.
6. Determine the absorption at 260 nm using approximately 1 μ L diluted to 1 mL with TE, and determine the concentration (Note 5).

3.3 Oligonucleotide Labeling

1. Mix 20 μ L of 0.1 μ M oligonucleotide, 5 μ L polynucleotide kinase buffer (10 X, supplied with enzyme), 2 μ L γ -³²P-ATP (10 μ Ci/ μ L, 6000 Ci/mmol), and 21 μ L water to make a total volume of 48 μ L and centrifuge briefly. Add 2 μ L T4 Polynucleotide Kinase (10 unit/ μ L) and mix by gentle tapping.
2. Incubate 37°C for 60 min.
3. Stop reaction by adding 2 μ L 0.5 M EDTA.
4. TCA analysis:
 - a. Remove 1 μ L from the reaction mixture to a 13 $\times$ 100 mm test tube containing 100 μ L of 100 μ g/mL salmon sperm DNA in TE. Mix well.
 - b. Spot 1 μ L of this mixture directly onto a Whatman GF/C 47 mm filter disk.
 - c. To remaining 100 μ L, add 5 mL ice cold 10% TCA, vortex, and leave on ice 15 min.
 - d. Collect precipitate by vacuum filtration through a GF/C filter. Wash the tube and filter 5 $\times$ 5 mL ice cold TCA, then 2 $\times$ 5 mL ice cold ethanol.
5. Analysis. Count both filters either by Cerenkov radiation (without scintillation fluid) or with 5 mL scintillation fluid. Total counts are obtained from the directly spotted 1 μ L by:

$$\text{Total} = \text{C.P.M.} \times (101 \mu\text{L}/1 \mu\text{L}) \times (52 \mu\text{L}/1 \mu\text{L})$$

And that precipitated:

$$\text{TCA} = \text{C.P.M.} \times (100 \mu\text{L}/101 \mu\text{L}) \times (52 \mu\text{L}/1 \mu\text{L})$$

The efficiency of labeling is determined from the percent of total counts incorporated into TCA precipitable DNA. The specific activity is just the TCA precipitate counts divided by the 2 pmol of oligonucleotide used for labeling.

6. Recovery. The oligonucleotide is desalted on a spin column. This treatment removes over 90% of the remaining γ -³²P-ATP. This can be done using a commercially available column. However, we make our own columns from a 1 mL tuberculin syringe barrel plugged with silanized glass wool and placed inside a 17 $\times$ 100 mm culture tube through a small hole made in the lid with a pair of scissors. (Figure 1). The column (syringe barrel) is filled with a 1:1 slurry of BioRad P-6 resin. The culture tube serves as a receptacle. The column is centrifuged until no more fluid elutes in an IEC clinical centrifuge at maximum speed for 10 min. The

⁵Alternatively, a nanodrop spectrophotometer can be used but results are less accurate.

eluate is discarded and a 1.5 mL centrifuge tube is placed under the column outlet, all inside the culture tube. The labeled DNA (51 μ L, step 3) is added to the column and the column is again centrifuged. The 50–55 μ L eluate is then ready for use. The oligonucleotide is now approximately 40 nM (Note 6).

7. The specific activity of the DNA is adjusted to 4,000 cpm/pmol, and 10 nM oligonucleotide and stored in 50 μ L aliquots in the -20°C freezer. For example, if the oligonucleotide is diluted 4-fold with TE, it is 10 nM. If labeling efficiency, calculated from TCA precipitable radioisotope (step 5) is 50%, the oligonucleotide is now 200 μ L and 66,000 dpm/ μ L. Further dilution with 3.1 mL of unlabeled 10 nM oligonucleotide in TE provides the desired specific activity. Normally, only sufficient 50 μ L aliquots are prepared for two weeks experiments and the rest discarded in liquid waste. The stored oligonucleotide is usable for two weeks for EMSA.

3.4 Electrophoretic mobility shift assay (EMSA)

1. Prepare a non-denaturing polyacrylamide gel. The following procedure is for 10 mL gel (enough for two 0.75 mm $\times$ 7 $\times$ 10 cm minigels). For most purposes the 5% gel is used. In a 16 $\times$ 100 mm test tube, combine:

	5%	10%	12%
H ₂ O	7.7 mL	6.1 mL	5.4 mL
5 X TBE	0.5 mL	0.5 mL	0.5 mL
Acrylamide: bis (29:1)	1.7 mL	3.3 mL	4 mL
10% Ammonium persulfate	0.1 mL	0.1 mL	0.1 mL
TEMED	20 μ L	20 μ L	20 μ L

2. Mix well and quickly pour into the assembled plates and place the comb. Let the gel polymerize (approximately 20 min).
3. Fill the gel tank with 0.25 X TBE (~300 mL).
4. Remove the comb, wash and fill the wells with 0.25 X TBE. Pre-electrophorese for 30 min at 100 V.
5. In a microtube, combine:

5 X EMSA buffer	5 μ L
1 μ g/ μ L Poly (dI-dC)	1 μ L
Nuclear Extract or purified fraction	2 μ L
10 nM ³² P-labeled, annealed oligonucleotide	5 μ L
H ₂ O	12 μ L

6. Mix gently and incubate at room temperature for 20 min.
7. Add 2 μ L of bromophenol blue to each sample, mix gently. Load onto gel.

⁶The centrifuge tube used for collection can be pre-weighed and weighed again after collection to determine the volume more accurately.

8. Gel electrophoresis. Fill the upper and lower chambers of the gel apparatus with 0.25 X TBE if this has been removed. Run the gel at 100 V for 50 min or until dye front is ~1 mm from the bottom of the gel.
9. Autoradiography. Dry the gel overnight or place in a water tight zip-lock bag. Expose film overnight at -70°C using an intensifying screen. A typical EMSA read-out for the CAAT enhancer binding protein transcription factor (C/EBP) is shown in Figure 2.

3.5 DNA-Sepharose Preparation (requires 2 days)

1. Using the oligonucleotide clean-up protocol (Section 3.2), prepare the oligonucleotides and dissolve in water. For trapping methods, $(\text{AC})_5(\text{NH}_2\text{-ACACACACAC})$, where “ NH_2 ” refers to the aminohexyl phosphoramidite added on the last cycle during synthesis, oligonucleotide is used. If beginning from a 1 μmol synthesis, approximately 500 nmol will be obtained. Coupling is with 50 nmol $(\text{AC})_5/\text{mL}$ Sepharose which is 175 nmol/g lyophilized CNBr Sepharose. Assuming 500 nmol is obtained, 10 mL of oligonucleotide-Sepharose can be prepared or the recipe adjusted to fit the amount available. Keeping the oligonucleotide volume as low as possible (less than 1 mL), place 500 nmol in a 50 mL sterile, conical, graduated tube kept on ice.
2. Weigh 2.9 g of lyophilized cyanogen bromide-activated Sepharose 4B into an ice-cold 250 mL graduated cylinder.
3. Fill graduated cylinder with 250 mL ice-cold 1 mM HCl, cover with Parafilm, and invert to mix. Let the Sepharose swell for 15 min while keeping the graduated cylinder on ice, mixing by inversion every few minutes. The graduated cylinder is used here because the Sepharose settles slowly over the long dimension, keeping it well suspended over the swelling process.
4. Filter and wash the Sepharose three times, each with 100 mL ice-cold 1 mM HCl on the coarse 600 mL Kimax sintered glass filter funnel, leaving a moist cake. It is important that the funnel be fast flowing, allowing the washes to be accomplished in 2–3 min.
5. Scrape the Sepharose into the DNA containing conical tube. Mix and add sufficient coupling buffer (approximately 20 mL) to produce an easily mixed, fairly liquid slurry.
6. Put the capped tube on a wheel rotator (Cole-Parmer Roto-Torque) overnight at room temperature. The tube is placed along the periphery of the wheel so that the solution rotates over the side wall of the tube. The rate of rotation is as slow as possible while keeping the Sepharose suspended.
7. On the next day, filter the Sepharose on a coarse 30 mL sintered glass funnel using a side-arm flask of known weight and wash the tube and Sepharose four times with 5 mL of blocking buffer.
8. Re-weigh the side-arm flask to determine the volume of the total filtrate plus washes (1 g = 1 mL), usually about 35–40 mL. As long as the volume is more than 10 mL, the $A_{260\text{ nm}}$ can be determined directly without dilution.
9. The nmol of DNA recovered is:

$$\text{nmol } (\text{AC})_5 \text{ NOT coupled} = \text{mLs recovered} \times 10^6 \times A_{260\text{ nm}} / 111,250$$

10. The nmol of DNA coupled is calculated from the results in steps 1 and 9 as:

$$\text{nmol (AC)}_5 \text{ coupled} = \text{nmol (AC)}_5 \text{ added} - \text{nmol (AC)}_5 \text{ not coupled}$$

11. Return the filtered Sepharose to the 50 mL screw-cap tube and add sufficient blocking buffer (about 20 mL) to again produce a liquid slurry. Blocking buffer reacts with any remaining activated groups on the Sepharose, rendering them inert.
12. Incubate on wheel rotator overnight at room temperature. Wash DNA-Sepharose two times with 4 mL TE0.1 (see section 2.2) containing 10 mM NaN₃ on the filter funnel. Return the Sepharose to a fresh 50 mL conical screw cap tube, and after the Sepharose has settled, add or subtract a sufficient amount of the same buffer to make an exact 1:1 slurry. Yield is about 10 mL of settled (AC)₅-Sepharose and about 20 mL of the 1:1 slurry. Typically, about 80–100% of the (AC)₅ couples, yielding about 25–50 nmol (AC)₅/mL Sepharose. The (AC)₅-Sepharose is stable at 4°C for at least one year.
13. To prepare a 1 mL column, a 1.5 mL column is outfitted with a frit at its outlet, a stopcock, and filled with TE0.1. Most of the TE is allowed to flow through the frit leaving approximately 0.2 mL in the column. One mL of well mixed 1:1 (AC)₅-Sepharose slurry is added, and flow is again initiated. As the column drains, a second mL of slurry is added, maintaining a full column until all is added. The column is then watched carefully while flowing and when the bed is no longer increasing, flow is stopped, usually leaving approximately 0.2 mL of clear buffer above the resin bed. A second frit is then placed carefully on top of the resin bed so that a flat top which cannot be easily disturbed is produced. The column is then outfitted with a stopper through which a 18 g × 1 in. needle is placed and an empty 10 mL syringe barrel is attached to the needle to serve as a reservoir. The column is then washed with 10 mL of TE0.1 to equilibrate the column and if necessary, the top frit is again adjusted to the top of the resin bed. For C/EBP trapping, the column is washed with a further 10 mL of TE0.4. For smaller columns (e.g., 0.1 mL), column barrels and frits recovered from a used QIAquick PCR purification column or Qiagen RNAeasy column are used.

3.6 Oligonucleotide Trapping Chromatography (Figure 3)

1. The oligonucleotide used for trapping depends upon the transcription factor sought and the DNA response element it binds. For C/EBP we used EP24 (without a tail) for EMSA and ACEP24 (containing the (GT)₅ single stranded tail) for trapping (6, 7).
2. Both oligonucleotides are self-complementary, and are annealed in a thermocycler for 5 min at 95°C, followed by a linear decrease to 4°C over the next hour.
3. For simple oligonucleotide trapping, trapping is modeled on conditions used for successful EMSA (Section 3.4) except using typically a TE based buffer for column loading and elution. For systematically optimized oligonucleotide trapping (7), EMSA assays are used to measure the DNA-binding affinity and concentration of the transcription factor in nuclear extract, and the optimal concentrations of substances such as NaCl, the single stranded oligonucleotide (dT)₁₈, heparin (a competitive inhibitor of DNA-binding), the double stranded DNA poly dI:dC, and detergents are determined. For C/EBP, the optimal conditions (Note 7) were: TE containing 0.4 M NaCl (TE0.4), 2.2 µg/mL HEK293 nuclear extract (Section 3.1), 1.34 nM annealed ACEP24(GT)₅, 930 nM (dT)₁₈, 50 ng/mL heparin, 50 µg/mL poly dI:dC, and 0.1% Tween-20. This mixture (50 mL) is allowed to equilibrate on

ice for 30 min to allow C/EBP to form a complex with the ACEP24(GT)₅ DNA. All subsequent steps are performed at 4°C.

4. The mixture is then applied to the 1 mL (AC)₅-Sepharose column (Section 3.5). After all 50 mL is applied, the column is washed with 20 mL of TE0.4, 0.1% Tween-20.
5. The column is then eluted with TE1.2 (TE containing 1.2 M NaCl) collecting 1 mL fractions.
6. The fractions containing C/EBP are then located using the EMSA assay and the purity is examined by SDS-PAGE. If necessary, the fractions can be concentrated using a Millipore Microcon Ultracell YM-10 centrifugal concentrator (Note 8).

3.7. Promoter Trapping Chromatography

1. Oligonucleotide trapping is used when the transcription factor binding to a specific binding site is being sought. Promoter trapping allows the purification of an active transcription complex binding to a promoter DNA sequence. As a proof-of-concept, we focus in this chapter on purifying the transcriptional complex of the 281 bp core promoter of *c-jun* (8).
2. Making the DNA promoter trap: the c-jun core promoter is produced by PCR and cloned into pUC19 to yield pUC19-c-jun core promoter plasmid (8) which is used as template for PCR. To produce the DNA with single-stranded (GT)₅ tails for trapping, the following protocol is used: two different PCR reactions are performed with the following primers (where “Phos” denotes a 5'-phosphorylated oligonucleotide produced during synthesis);

FP	5'-cgggatcccagcggagcattactc-3'
RP	5'-cggaattcgctgctgtgtgtgtgtc-3'
(AC) ₅ FP	5'-Phos/acacacacagcgatcccagcggagcattacc
(AC) ₅ RP	5'-Phos/acacacacgaattcgctgctgtgtgtgtc

Reaction 1 utilizes FP and (AC)₅RP while reaction 2 utilizes (AC)₅FP and RP and the reactions are performed separately. PCR (50 µL) containing 200 nM primers, 10 ng of pUC19-c-jun core promoter, 1.25 mM MgCl₂, 250 µM dNTP mixture and 1 U Taq DNA polymerase in PCR buffer (New England Biolabs) is heated at 95°C for 5 min, thermocycled for 1 min at 95°C, 1 min at 50°C, and 2 min at 72°C for 35 cycles, followed by 10 min at 72°C for extension. 250 µg of each PCR product, obtained from multiple replicate reactions, is purified using the QIAquick PCR purification columns (Qiagen) and eluted in 2 mL TE buffer. The resulting 250 µg for each reaction type (1 or 2) is then digested using the supplier's protocol with

⁷Determination of optimal concentrations is complex and more fully explained elsewhere (7). Essentially, the EMSA assay is used to determine the apparent dissociation constant of the protein for the DNA used (K_d) and the maximal amount of the binding protein (B_{max}) in the nuclear extract. Then, using nuclear extract containing an amount of protein equal to K_d and a concentration of DNA equal to ten times K_d is used for all subsequent EMSA assays and for the final purification. Beginning with dI:dc, different concentrations are added to the EMSA assay to determine the highest concentration that can be used without inhibiting binding. This concentration is then fixed for all subsequent assays. Similarly, the highest non-inhibiting concentration of (dT)18, heparin, and detergent is then tested and set at this concentration for all subsequent assays. The final resulting optimal concentrations are then used for oligonucleotide trapping purification.

⁸Occasionally, the concentrator is leaking. It is therefore best to retain the filtrate and confirm by absorption (280 nm) that protein has been removed.

100 units of lambda exonuclease for 2 h at 37°C. Lambda exonuclease digests single strands containing a 5'-phosphoryl end to nucleotides, and since reactions one and two only have a single phosphorylated strand, the result is two single-stranded DNAs which are complementary. The two strands are then mixed and annealed (section 3.6 step 2).

3. The annealed DNA contains the duplex c-jun core promoter (−200 to +81 bp) with a 3'-(GT)₅ single-stranded tail on each strand. The annealed DNA is purified by applying it (now approximately 500 µg duplex) to a fresh 1 mL (AC)₅-Sephacrose column equilibrated in TE0.1 at 4°C. The column is then washed with 20 mL TE0.1 at 4°C, moved to room temperature and then eluted with 37°C TE containing 0.1% Tween-20, collecting 0.5 mL fractions (Note 9). Fractions are analyzed by agarose gel electrophoresis and fractions containing duplex c-jun promoter DNA are combined; the concentration is determined by absorption at 260 nm (assuming 50 µg/mL DNA has an absorbance of 1.0) and stored frozen at −20°C.
4. 100 µL HEK293 nuclear extract (0.5 mg nuclear protein) is diluted to a final volume of 1 mL with TE0.1 buffer containing 0.1% Tween-20, poly dI:dC (30 µg/mL final) and incubated for 10 min at 4°C. The tailed c-jun (GT)₅ (calculated molecular weight 187,488) is then added to a final concentration of 60 nM and incubated to form a complex for 30 min at 4°C. At 4°C, the mixture is applied to a 0.1 mL (AC)₅-Sephacrose column, washed with 20 column volumes of TE0.1 containing 0.1% Tween-20, and proteins bound on the column are eluted with TE0.4 buffer. Samples from TE0.4 elution are dialyzed in 50 mM NH₄HCO₃ and lyophilized (Note 10).

3.8 Two-dimensional gel electrophoresis (2DGE) and blotting

1. Isoelectric focusing (IEF) is performed with ReadyStrip IPG strips (pH 3–10, linear, 7 cm) using the PROTEAN IEF cell (BioRad) according to the manufacturer's protocol. HEK293 nuclear extract (100 µg) or a similar amount obtained from oligonucleotide trapping or promoter trapping is mixed in 125 µL rehydration buffer and rehydrated at 50 V for 16 h. IEF is then performed at 40,000 V.h at 20°C.
2. The strips are equilibrated in 2.5 mL equilibration buffer containing 2% DTT at room temperature for 15 min. The strips are then removed and incubated in 2.5 mL equilibration buffer containing 2.5% iodoacetamide in the dark for 15 min. The strips are transferred to 12% SDS-PAGE gels for a second dimension of electrophoresis using the PROTEAN II xi 2-D (BioRad) cell at constant 10 mA/gel for 2 h. After electrophoresis, the gel is stained with silver nitrate or transferred to NC or PVDF membrane for Western blotting (WB) or Southwestern blotting (SW) analysis.
3. Gel blotting is performed as described (9) with minor modifications. Briefly, the protein sample, separated by SDS-PAGE or 2DGE, is transferred to PVDF membrane at 110 V for 1.5 hr in the cold room in blotting buffer. For Southwestern blotting, PVDF gives the best performance and is used in Figure 4.

⁹The promoter DNA binds to the column by annealing of the (GT)₅-tail with (AC)₅-Sephacrose in TE0.1 at 4°C. This is disrupted in low salt (TE) at elevated temperature. The duplex promoter, because of its length, is unaffected.

¹⁰The complex isolated has been shown to be transcriptionally active and to contain specific transcription factors, the TFII complex, and RNA polymerase 2 (8). Lyophilization may affect this activity but not further analysis by Western blot or Southwestern blot.

3.9 2DGE-Southwestern blotting (2DGE-SW)

1. The blotted proteins are denatured and renatured by immersing the blot in 10 mL 6 M guanidine HCl, which is then serially diluted to 3, 1.5, 0.75, 0.375, 0.188, 0.094 M using 1X binding buffer with incubation at 4°C for 10 min each time.
2. The blot is blocked at room temperature for one hour in 1X binding buffer containing 0.5% polyvinyl pyrrolidone (PVP40) for 30 min. Alternatively, 5% milk in 1X binding buffer has been used, blocking overnight at 4°C (Note 11).
3. The membrane is probed overnight for C/EBP with [γ -³²P] radiolabeled EP24 (1.5 nM, 10⁶ cpm/mL) or with radiolabeled 2 nM 281 bp c-jun core promoter (for nuclear extract or promoter trapping fractionated nuclear extract) or with various other radiolabeled oligonucleotides (unpublished) at 4°C in 1X binding buffer containing 0.25% BSA and 10 µg/mL poly dI:dC. The washed membrane is air-dried and exposed to film for 12 h for autoradiography. Both a Western blot (upper panel) and Southwestern blot (lower panel) is shown in Figure 4B.

3.10 On-blot trypsin digestion for ESI-MS analysis

1. The protein spots located by 2DGE-SW are excised (typically a 1–2 mm circle) and stripped at 50°C with SDS stripping buffer for 10 min, and then the membrane is washed ten times with 500 µL H₂O for 1 min/change. This stripping effectively removes the radiolabeled DNA probe and also effectively reduces interference from components used for blocking the membrane.
2. The blotted protein is reduced with 10 mM DTT at 56°C for one hour, and then alkylated by 55 mM iodoacetamide at room temperature in the dark for one hour. After washing with 500 µL 25 mM NH₄HCO₃ five times for 1 min, the blot is immersed in 20 µL of 25 mM NH₄HCO₃ and heated at 95°C for 5 min.
3. 5 µL of 5% Zwittergent 3–16, 15 µL acetonitrile (ACN), and 10 µL of 40 µg/mL trypsin are added to a final volume of 50 µL, and the blot digested overnight at 37°C. The blot in digestion solution is sonicated (in a bath sonicator) for 5 min, briefly centrifuged, and the supernatant collected. The peptides on the membrane are further extracted with 30 µL of 5% trifluoroacetic acid (TFA)/50% ACN at room temperature for 2 h and then 30 µL 0.5% TFA/50% ACN for another 2 h. The combined eluate (110 µL) is vacuum dried and dissolved in 30 µL of 0.1% TFA. The eluate is again vacuum dried.
4. The eluate is dissolved in 10 µL 0.1% TFA prior to LC-nanosprayESI-MS/MS. Most scientists have available to them an excellent proteomics facility for analysis, but commercial analysis could also be an option. Therefore, this is likely to be the last step for most investigators. However, we often perform our own analysis, detailed below, which may serve as a model for analysis.

3.11 Capillary HPLC-Electrospray Tandem Mass Spectrometry (LC-nanosprayESI-MS/MS)

1. Analysis is on a Thermo Finnigan LTQ linear ion trap mass spectrometer equipped with a nano-ESI source.

¹¹Which blocking procedure is chosen depends on subsequent experiments. PVP-40 fragments in the mass spectrometer to give a complex spectrum but works best for renaturation and retention on the blot and gives the highest signal for Southwestern blotting. Bovine serum albumin is often not advised by commercial mass spectrometry facilities. If the protein spots are going to be used directly after this step for mass spectrometry, some interference by either method would be expected. However, in section 3.10 step 1, the blocking medium is stripped away and probably neither blocking approach would significantly interfere. We have successfully used both when the complete procedure given here is used for analysis.

2. On-line HPLC separation of the digests is accomplished with an Eksigent HPLC micro HPLC. The column is a PicoFrit Emitter (New Objective; 50 μm i.d., 15 μm tip) packed to 10 cm with C18 adsorbent (Alltech Altima, 5 μm , 300 $\text{\AA}$). The gradient is from 2 to 42% mobile phase B in 30 min. The flow rate is 0.3 $\mu\text{L}/\text{min}$. MS conditions are a 2.5 kV ESI voltage, an isolation window for MS/MS of 3, 35% relative collision energy, with a scan strategy of a survey scan followed by acquisition of data dependent collision-induced dissociation (CID) spectra of the seven most intense ions in the survey scan above a set threshold.
3. Database searching is with Mascot software (Matrix Science, Boston, MA, USA, version 2.1.0) against either the Swiss-Prot 49.0 (268,833 sequences, 123,649,849 residues) or our in-house human transcription factor database. The search parameters are: trypsin digestion, two possible missed cleavages, monoisotopic mass values, peptide mass tolerance of 1.0 Da., MS/MS tolerance 0.8 Da., instrument ESI-TRAP and oxidized methionine and cysteine carboxyamidation as variable modifications. Results were scored based on the probability based Mowse score. The score threshold to achieve $p < 0.05$ is set by Mascot and is based on the size of the database searched. Proteins with a probability less than threshold and with two or more independent peptide sequences are considered as true positives. The on-blot digestion and MS/MS characterization of C/EBP- β from HEK293 nuclear extract is shown in Figure 4C.

Acknowledgments

We thank Dr. William Haskins and the UTSA RCMi Proteomics and Protein Biomarkers Core facility for help with analysis and the use of their equipment. We also appreciate the help of UTSA RCMi Bioinformatics and Computational Biology core for computing. We also thank Daifang Jiang for reading the manuscript and much helpful discussion. This work was supported by NIH grant R01GM043609 and a grant from the San Antonio Life Sciences Institute.

References

1. Hahn S. Structure and mechanism of the RNA polymerase II transcription machinery. *Nat Struct Mol Biol.* 2004; 11:394–403. [PubMed: 15114340]
2. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal

A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A, Zhu X. The sequence of the human genome. *Science*. 2001; 291:1304–1351. [PubMed: 11181995]

3. Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM. A census of human transcription factors: function, expression and evolution. *Nature Reviews Genetics*. 2009; 10:252–263.
4. Abmayr, SM.; Yao, T.; Parmley, T.; Workman, J. *Current Protocols in Molecular Biology*. John Wiley & Sons; 2006. Preparation on Nuclear and Cytoplasmic Extracts from Mammalian Cells; p. 12.11
5. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
6. Gadgil H, Jarrett HW. Oligonucleotide trapping method for purification of transcription factors. *J Chromatogr A*. 2002; 966:99–110. [PubMed: 12214709]
7. Moxley RA, Jarrett HW. Oligonucleotide trapping method for transcription factor purification systematic optimization using electrophoretic mobility shift assay. *J Chromatogr A*. 2005; 1070:23–34. [PubMed: 15861784]
8. Jiang D, Moxley RA, Jarrett HW. Promoter trapping of c-jun promoter-binding transcription factors. *J Chromatogr A*. 2006; 1133:83–94. [PubMed: 16934821]
9. Ohri S, Sharma D, Dixit A. Interaction of an approximately 40 kDa protein from regenerating rat liver with the –148 to –124 region of c-jun complexed with RLjunRP coincides with enhanced c-jun expression in proliferating rat liver. *Eur J Biochem*. 2004; 271:4892–4902. [PubMed: 15606777]

Figure 1.
A cross-section of a desalting spin column is shown.

Figure 2.

Electrophoretic mobility shift assay of rat liver nuclear extract C/EBP. A two-fold serial dilution of rat liver nuclear extract (220 ng/ μ L stock) was incubated with 1.6 nM radiolabeled ACEP24(GT)₅. The fold dilution is shown above the gel. Poly dI:dC was constant at 40 μ g/mL. C, specific shift complex identified by other experiments; U, unshifted double stranded DNA; NP, no protein added to the gel (Footnote 1).

¹Reproduced from R.A. Moxley and H.W. Jarrett, "Oligonucleotide trapping method for transcription factor purification systematic optimization using electrophoretic mobility shift assay", *The Journal of Chromatography A* 1070 (2005) 23-34, , Copyright 2005, with permission from Elsevier."

Figure 3.

Silver (Ag) stain and western blot (WB) of oligonucleotide trapping fractions of C/EBP (using ACEP24 oligonucleotide) from rat liver nuclear extract. Carbonic anhydrase (31 kDa) and ovalbumin (42 kDa) molecular masses are indicated. NE, rat liver nuclear extract. Fraction F1 was collected starting as TE1.2 eluted the column. Silver staining of the TE1.2 elution fraction (F1) shows two highly purified and isolated proteins of similar relative molecular weight as C/EBP- α isoforms. A western blot using 1:100 sc-61 (Santa Cruz Biotechnology, 14AA anti-C/EBP- α antibody) demonstrates the presence of C/EBP- α in rat liver nuclear extract, and confirms the isolation and purification of C/EBP- α using the oligonucleotide trapping method. 1 μ g of NE was added in both the silver stain and western blot where indicated. For fraction F1, 1/5 of the total fraction was used for electrophoresis. For Western blot of F1, 1/20 of total fraction was used (Footnote 1).

Figure 4. 2DGE-SW analysis of HEK293 nuclear extract. **(A)** HEK293 nuclear extract was separated by 2DGE. One 2DGE gel (50 µg nuclear extract) was stained with silver nitrate in panel A. Encircled is the region of interest identified by Western and Southwestern blot as shown in the other panels. **(B)** Two 2DGE gels (100 µg nuclear extract each) were transferred to PVDF membrane for Western blot (upper panel) and Southwestern blot (lower) analysis in panel B. The two protein spots that reacted with the C/EBP antibody (Santa Cruz C/EBP-β antibody Δ198) and radiolabeled EP24 are indicated as “a” and “b”. The 2DGE-SW spots were analyzed by on-blot digestion and HPLC-nano-ESI-MS/MS analysis. Spot “a” was successfully identified as human C/EBP-β, as shown in panel C. **(C)** MS/MS analysis of 2DGE-SW spot “a”. MS/MS results shows two unique peptides from spot “a” matched with human C/EBP beta (NP-005185) with 9% sequence coverage after searching the local transcription factor database fused with the SwissProt database. Identification as C/EBP-β has a $P < 0.0035$. The mass spectrum shown below represent the MS/MS results of the doubly charged peptide AKMRNLETQHK at m/z 686.15 (2+). The matched b- and y-ions, derived from the peptide MS/MS, are indicated with arrows. The amino acid sequence of the parent peptide is shown on the top of the spectrum (Footnote 2).

²Reproduced from D. Jiang, Y. Jia, Y.W. Zhou, and H.W. Jarrett, "Two-dimensional southwestern blotting and characterization of transcription factors on-blot", *Journal of Proteome Research* 8 (2009) 3693-3701, Copyright 2009, American Chemical Society with permission from the publisher.