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Southwestern Blotting Assay

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Abstract

Southwestern blotting is a technique used to study DNA-protein interactions. This method detects specific DNA-binding proteins by incubating radiolabeled DNA with a gel blot, washing, and visualizing through autoradiography. A blot resulting from 1-dimensional SDS-PAGE reveals the molecular weight of the binding proteins. To increase separation and determine isoelectric point a 2-dimensional gel can be blotted. Additional dimensions of electrophoresis, such as a gel shift (EMSA), can precede isoelectric focusing and SDS-PAGE to further improve separation. Combined with other techniques, such as mass spectrometry, the DNA-binding protein can be identified.

Keywords

Transcription; Regulation; DNA; Response elements; Oligonucleotide; Promoter; Immunoblots; Nitrocellulose membrane; PVDF membrane; Southwestern blotting

1 Introduction

Regulation of gene expression is essential in human development as well as pathogenesis [1]. The gene regulatory mechanism involves distinct 6–8 bp DNA motifs referred to as response elements, which bind transcription factors [2]. Transcription factors are DNA-binding proteins that interact with unique response elements at the promoter region of DNA, or other functional cisacting response elements, resulting in either gene expression or repression [1]. Identification of transcription factors specific to a particular gene is not only significant to gene regulation but also to understanding gene function. Southwestern blotting assay (SWB) is one of the most powerful techniques to explore protein-DNA interaction and transcription factor regulation.

SWB, similar to other blotting techniques, separates proteins (or DNA) by gel electrophoresis. The gel containing the separated proteins is electro-transferred (blotted) to a membrane, such as nitrocellulose (NC) or polyvinylidene difluoride (PVDF). To detect the DNA-binding proteins, the proteins are partially renatured and bound to nanomolar concentrations of radiolabeled DNA. Any unbound DNA is washed away, and then bands on the blot are detected by autoradiography. This technique is especially useful in the identification of transcription factor as it gives information on the molecular weights of all DNA-binding proteins involved with a particular sequence of DNA. An experimental procedure is shown schematically in Fig. 1.

Since the original SWB assay, first described in 1980 [3], many extensions of the SWB method have been developed [4, 5]. In this chapter, three detailed SWB methods are described that differ in the number of electrophoresis dimensions used for protein separation. 1D-SWB uses separation on a single SDS-PAGE dimension, 2D-SWB uses separation by isoelectric focusing followed by SDS-PAGE in the second dimension, while 3D-SWB uses separation by DNA gel shift on a non-denaturing gel before 2D-SWB. Coupled with proteomic techniques, several transcription factors have been identified [4]. Additionally, a method has been developed that allows multiple reprobes with different oligonucleotides on one blot [6]. Each of these SWB techniques provides alternatives to investigate regulatory transcription factors in vitro.

2 Materials

2.1 Oligonucleotide Preparation

1. Oligonucleotide: Approximately 20–25 nt in size along with its complementary (antisense) strand, containing one protein-binding motif (0.1 μ mol).
2. TE buffer: 10 mM Tris (free base), titrated to pH 7.5 with HCl, 1 mM EDTA.
3. 0.5 M EDTA (free acid): Titrated with 5 M NaOH to pH 8.0.
4. 3 M Sodium acetate solution: Dissolve 40.8 g sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$) in water to a final volume of 100 mL, and adjust to pH 5.2 with glacial acetic acid.

2.2 Oligonucleotide Labeling

1. ATP, [γ - ^{32}P]: 6000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{L}$, 250 μCi , stored at -20°C .
2. T4 Polynucleotide Kinase: 10 U/ μL , store at -20°C .
3. 10 % Trichloroacetic acid (TCA): 10 g TCA dissolved to 100 mL in H_2O (see Note 1).
4. Silanized glass wool.
5. Culture tubes: Sterile, 17 $\times$ 100 mm sterile culture tubes.
6. Bio-Gel P-6 Fine Resin: Suspend 10 g of resin (Bio-Rad Laboratories, Hercules, CA, USA) in 250 mL of TE (pH 7.5) and autoclave for 45 min. Cool to room temperature, and then wash the resin with fivefold resin volumes TE three times. Remove excess liquid to give 1:1 slurry.

2.3 Cell Culture and Nuclear Protein Preparation

1. Human embryonic kidney 293 cells (HEK293).
2. Cell culture flasks: 182 cm^2 .
3. Dulbecco's modification of Eagle's medium (DMEM).

¹Water is $>18\text{ M}\Omega$ from a Millipore Synergy UV water purification unit.

4. Adult bovine serum: The serum is inactivated by incubation at 56 °C for 30 min and stored at 4 °C prior to use.
5. 10× PBS: 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ made up to 1 L with H₂O and autoclaved prior to use.
6. 1 M Dithiothreitol (DTT): Dissolve 15.4 g DTT in 100 mL of water. Aliquot and store at −20 °C for up to 6 months.
7. 1× Trypsin-EDTA.
8. Phenylmethylsulfonyl fluoride (PMSF, 0.2 M): Dissolve 3.48 g PMSF in 100 mL of anhydrous isopropanol as a 0.2 M stock. Stored at −20 °C for up to 1 year.
9. Nuclear extract hypotonic buffer: 10 mM HEPES, pH 7.9 at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 0.5 mM DTT (added prior to use), and 0.2 mM PMSF (added prior to use).
10. Nuclear extract low-salt buffer: 20 mM HEPES, pH 7.9 at 4 °C, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, and 0.2 mM PMSF (both added prior to use).
11. Nuclear extract high-salt buffer: 20 mM HEPES, pH 7.9 at 4 °C, 1.5 mM MgCl₂, 1.6 M KCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, and 0.2 mM PMSF (both added prior to use).
12. Nuclear extract dialysis buffer: 20 mM HEPES, pH 7.9 at 4 °C, 100 mM KCl, 20 % glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF (both added prior to use).

2.4 Electroblothing and SWB assay

1. Electroblothing buffer: 20 % (v/v) methanol, 25 mM Tris-base, 192 mM glycine.
2. SWB buffer: 10 mM HEPES (pH 7.9 at 4 °C), 50 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 50 μM ZnSO₄, and 0.1 % (v/v) Tween-20.
3. SWB blocking buffer: 5 % of nonfat dry milk in SWB buffer.
4. 6 M Guanidine hydrochloride: Dissolve 57.3 g guanidine hydrochloride (for molecular biology, 99 %) in 50 mL of SWB buffer, and then adjust to total 100 mL, freshly prepared prior to use.

2.5 1-Dimensional Gel Electrophoresis SWB (1DGE-SWB)

1. 30 % Acrylamide/Bis: Dissolve 29.2 g acrylamide and 0.8 g *N,N'*-methylene-bis-acrylamide (Bis) to a final volume of 100 mL H₂O.
2. 0.5 M Tris-HCl, pH 6.8: Dissolve 60.6 g Tris base in 800 mL H₂O, titrate to pH 6.8 with 1 M HCl, and adjust to 1 L with H₂O.
3. 1.5 M Tris-HCl, pH 8.8: Dissolve 181.7 g Tris base in 800 mL H₂O, titrate to pH 8.8 with concentrated HCl, and adjust to 1 L with H₂O.

4. 50 % Glycerol, 0.01 % bromophenol blue (BPB): To 50 mL glycerol, add 10 mg BPB, and adjust to 100 mL with H₂O.
5. 1 M Tris, pH 7.5: Dissolve 121.1 g Tris base in 800 mL H₂O. Titrate to pH 7.5 with concentrated HCl. Adjust volume to 1 L with H₂O and autoclave for 45 min.
6. 10 % (w/v) Ammonium persulfate (APS): Dissolve 100 mg APS (Sigma) in 1 mL H₂O, prepared prior to use.
7. β -Mercaptoethanol.
8. Tetramethylethylenediamine (TEMED).
9. 10 % (w/v) Sodium dodecylsulfate (SDS): Dissolve 10 g of SDS in H₂O to a final 100 mL; store at room temperature.
10. 5 $\times$ Laemmli sample buffer: Mix 0.12 mL #2: 0.5 mL #4: 0.05 mL #7:0.2 mL #9 on the day of use.
11. 10 $\times$ Running buffer: 250 mM Tris base, 1.92 M glycine. Working solution is prepared with 30 mL 10 $\times$ and 3 mL 10 % SDS and adjusted to 300 mL with H₂O and should be pH 8.3.

2.6 2-Dimensional Gel Electrophoresis SWB (2DGE-SWB)

1. Immobilized pH gradient (IPG) strip: (Bio-Rad) Ready@Strip™ IPG strips, linear 7 cm, pH 3–10.
2. Rehydration buffer: 7 M Urea, 2 M thiourea, 2 % CHAPS, 65 mM DTT, 0.2 % Bio-Lyte 3/10 Ampholytes (Bio-Rad), 1 % Zwittergent 3–10 (Sigma), and 0.001 % bromophenol blue.
3. Equilibration buffer (EB): 50 mM Tris, pH 8.8, 6 M urea, 2 % (w/v) SDS, 30 % (v/v) glycerol, and 0.001 % (w/v) bromophenol blue.
4. Reduction buffer: 2 % (w/v) DTT in EB.
5. Alkylation buffer: 2.5 % (w/v) Iodoacetamide in EB.

2.7 EMSA-Based SWB (3DGE-SWB)

1. 5 $\times$ EMSA buffer: 100 mM HEPES (pH 7.9), 0.5 mM EDTA, 250 mM NaCl, 25 mM MgCl₂, 5 mM DTT, 50 % (v/v) glycerol, and 0.5 % (v/v) Tween-20. Store at –20 °C for 1 month.
2. Poly (deoxyinosinic-deoxycytidylic) acid (poly-dI:dC): (Sigma) Dissolved in TE to bring to 0.5 mg/mL stock. Stored at –20 °C for at least 3 months.
3. 5 $\times$ Loading buffer: 50 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue.
4. 5 $\times$ TBE buffer: 54.5 g Tris base, 27.8 g boric acid, 3.7 g Na₄EDTA, pH 8.3 at room temperature.
5. Extraction buffer: 50 mM Tris, pH 9.0, 50 mM DTT, and 0.5 % (v/v) Tween-20.

3 Methods

3.1 Oligonucleotide Preparation

1. Dissolve received oligonucleotide in 300 μ L of TE (pH 7.5) buffer (*see* Note 2).
2. Add 30 μ L of sodium acetate solution (3 M) and 1 mL of 100 % ethanol (absolute), mix, and allow oligonucleotides to precipitate at -85°C for 1 h (*see* Note 3).
3. Collect oligonucleotides by centrifugation at $14,000 \times g$ using bench centrifuge for 15 min at 4°C (*see* Note 4).
4. Carefully remove the supernatant and wash the pellet with 500 μ L of ice-cold 70 % (v/v) ethanol by vortex mixing.
5. Again, centrifuge and remove the supernatant as in **step 4**.
6. Air-dry the oligonucleotide by leaving the tube uncapped and covered with Kimwipes for 2 h at room temperature.
7. Dissolve the pellet in 500 μ L of TE (pH 7.5) at room temperature for 30 min with occasional vortex mixing.
8. Determine the concentration by measuring the absorption at 260 nm using 1 μ L oligonucleotide diluted to 1 mL water (*see* Note 5).
9. Adjust the concentration to 0.1 mM with TE (pH 7.5).

3.2 Oligonucleotide Labeling

1. Caution: All steps must be carried out in a radioactivity control area.
2. Mix 2 μ L of 10 μ M oligonucleotide, 5 μ L of polynucleotide kinase buffer (10 $\times$, supplied with enzyme), 2 μ L of γ - ^{32}P -ATP (10–20 μ Ci), and 2 μ L of T4 Polynucleotide Kinase (10 U/ μ L), and bring to 50 μ L with water in a 1.5 mL Eppendorf tube. Mix by gentle tapping; centrifuge briefly.
3. Incubate at 37°C for 60 min.

²Oligonucleotides are purchased from Integrated DNA Technologies (Coralville, IA, USA). Molar absorptivity ($E_{260\text{nm}}$) is provided to calculate concentration.

³For short oligonucleotides (<15 nt.), increasing ethanol to 1.2 mL and using 75 % ethanol for the washes may increase yield.

⁴Observe the tube orientation so that the position of the pelleted DNA, which may not be visible, is known and can be avoided.

⁵Alternatively, a NanoDrop spectrophotometer can be used; however the results may be less accurate.

- a. The oligonucleotide can be desalted on a commercially available column or they can be made in-house as shown in Fig. 5. Start by plugging the outlet of a 1 mL tuberculin syringe barrel with silanized glass wool.
- b. Fill the syringe barrel with 1 mL of 1:1 slurry of Bio-Gel P-6 resin.
- c. The column is placed inside a 17×100 mm culture tube with a hole punctured through the lid (scissors work well) to collect eluate from the column.
- d. Centrifuge the column at $2000 \times g$ for 5 min. If unsure about the time, repeat centrifugation for additional time until no further liquid elutes.
- e. Discard the eluate and add the labeled DNA to the column. Place a 1.5 mL Eppendorf tube (without lid) under the column and replace in the 17×100 mm carrier tube.
- f. Centrifuge the column and collect the eluate containing labeled DNA into a clean 1.5 mL centrifuge tube.

4. Stop reaction by adding 2 μL of 0.5 M EDTA.

To carry out TCA analysis remove 1 μL reaction mixture from **step 4** to a 13×100 mm test tube containing 100 μL of 100 $\mu\text{g/mL}$ salmon sperm DNA in TE. Mix well.

Spot 1 μL of this mixture directly onto a Whatman GF/C 47 mm filter disk.

To the remaining 100 μL , add 5 mL of ice-cold 10 % TCA, vortex, and leave on ice for 15 min.

Collect precipitate by vacuum filtration through a GF/C filter. Wash the tube and filter five times with 5 mL of ice-cold TCA, and then twice with 5 mL of ice-cold ethanol.

5. Count both filters by Cherenkov radiation (without) or with 5 mL of scintillation fluid. Total counts are obtained from the directly spotted 1 μL (**step 6**) 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 (**step 8**):

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

$$\text{Labeling efficiency} = [\text{CPM (from TCA)}/\text{CPM (Total)}] \times 100 \%$$

6. The oligonucleotide is desalted on a spin column (commercially available). The column is centrifuged for 5 min at $2000 \times g$ with a countertop centrifuge. The concentration of the oligonucleotide is now approximately 400 nM (*see Note 6*).
7. Equal amount of sense and antisense oligonucleotides are mixed and annealed by heating to 95 $^{\circ}\text{C}$ for 5 min, and then slowly cooling to room temperature over 1 h, or using a thermo-cycler annealing program.
8. The specific activity of the oligonucleotide is adjusted to 4,000,000 cpm/mL and 10 nM labeled oligonucleotide with TE buffer and/or unlabeled oligonucleotide, and then stored in 50 μL of aliquots at -20°C (*see Note 7*).

3.3 Nuclear Extract Preparation 7

1. Culture human embryonic kidney 293 (HEK293) cells in a NuAire IR Autoflow CO_2 water-jacketed incubator at 37 $^{\circ}\text{C}$ with 5 % CO_2 and 95 % atmospheric air. Seed 182 cm^2 cell culture flasks with 5×10^6 HEK293 cells and grow in 60 mL/flask of DMEM containing 10 % heat-inactivated adult bovine serum. For a typical preparation, we grow 5–10 flasks.
2. Grow cells to 90 % confluence.

⁶Desalting removes over 90 % of the remaining $\gamma\text{-}^{32}\text{P}\text{-ATP}$. The Eppendorf tube used for collection can be pre-weighed and weighed again after collection to determine the volume more accurately.

⁷If labeling efficiency (Subheading 3.2, **step 5**) is 50 %, after desalting the DNA will be 50 μL of 400 nM oligonucleotide, and contain 20×10^6 cpm. In this case, the 50 μL labeled oligonucleotide would be diluted to 2 mL with TE, resulting in 10 nM oligonucleotide. To this would be added 3 mL 10 nM unlabeled oligonucleotide (in TE) to give a total of 5 mL and 4×10^6 cpm/mL. Generally, enough 50 μL aliquots are prepared for 2 weeks of experiments and the rest discarded in liquid waste.

3. Rinse the cells with 1× PBS and then harvest by adding 10 mL of trypsin-EDTA solution for 5 min at 37 °C.
4. Immediately add 10 mL of DMEM containing 10 % inactivated adult bovine serum to stop trypsin.
5. Remove the cells from the flask and suspend in 40 mL 4 °C DMEM containing 10 % inactivated adult bovine serum. This method is a minor modification of the method of Abmayr et al. [7]. Keep all subsequent steps at 4 °C.
6. Centrifuge the cells ($1850 \times g$ for 5 min, 4 °C) in 50 mL disposable, sterile plastic conical tubes and wash with 50 mL PBS. Carefully remove the supernatant and note the packed cell volume (pcv) (*see* Note 8).
7. Quickly resuspend the cells in five pcv of ice-cold nuclear extract hypotonic buffer, centrifuge the cells ($1850 \times g$ for 5 min, 4 °C), and discard the supernatant.
8. Resuspend the cell pellet in three pcv of nuclear extract hypotonic buffer and swell for 10 min on ice.
9. Transfer the cells to an ice-cold Dounce homogenizer and homogenize with the type B pestle using ten slow up-and-down strokes.
10. Collect the nuclei by centrifugation ($3300 \times g$, 15 min, and 4 °C) in a graduated conical tube. Discard the supernatant and note the packed nuclear volume (pnv).
11. Add half pnv of nuclear extract low-salt buffer. Gently mix, and add dropwise half pnv of nuclear extract high-salt buffer, gently stirring for 30 min on ice.
12. Transfer the extracted nuclei to a JA-20 centrifuge tube and centrifuge at high speed ($25,000 \times g$) for 30 min. Save the supernatant and discard the pellet.
13. 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 (*see* Note 9).
14. Determine the protein concentration using Bradford protein assay and dilute as needed with fresh dialysis buffer for a final concentration of 5 mg/mL protein (approximately 1–2 mL final volume).
15. Centrifuge the dialyzed nuclear extract ($25,000 \times g$, 20 min), carefully aliquot 50–100 µL of the supernatant into cooled 1.7 mL Eppendorf tubes, and store at –85 °C for up to 1 year (*see* Note 10).

3.4 1-Dimensional Gel Electrophoresis SWB (1DGE-SW)

1. Pour 12 % polyacrylamide mini-gel with a Bio-Rad Mini-PROTEIN empty cassette gel casting system, as per the manufacturer's protocol (*see* Note 11).

⁸The pcv is approximately 4 mL (from ten of 182 cm² cell culture flasks) and contains 4×10^8 cells.

⁹Long dialysis is detrimental to activity. The dialysis procedure described preserves activity and thorough dialysis.

¹⁰Nuclear extract can be stored for at least 1 year at –85 °C.

¹¹The percentage of SDS-PAGE used is dependent on the molecular weight of the protein of interest. For two 12 % polyacrylamide SDS-PAGE mini-gels mix 4 mL of 30 % acrylamide/BIS solution, 2.5 mL of 1.5 M Tris–HCl, pH 8.8, 5 µL of TEMED, 50 µL of

2. Mix 20 μ L of protein sample and 5 μ L of Laemmli buffer (5 $\times$) and heat at 95 $^{\circ}$ C for 5 min to denature proteins.
3. Load 25 μ L of sample in each well plus one well for the molecular mass markers (*see* Note 12).
4. Fill electrophoresis cell with running buffer (1 $\times$ containing 0.1 % SDS) and electrophorese at 100 V for $\sim$ 1.5 h at room temperature until the BPB tracking dye is within 2–3 mm from the bottom.
5. Electroblot protein on the gel to PVDF membrane at 4 $^{\circ}$ C for 2 h with 100 mA in electroblotting buffer, using Bio-Rad electroblotting system.
6. Incubate blot in 25 mL of 6 M of guanidine solution for denaturing for 10 min.
7. Renaturing is by serially diluting the guanidine HCl solution with SWB buffer to a final concentration of 3, 1.5, 0.75, 0.375, 0.188, and 0.094 M. Between each dilution incubate the blot for 10 min at 4 $^{\circ}$ C (*see* Note 13).
8. Wash blot twice with 25 mL of SWB buffer, and place blot in 50 mL of SWB blocking buffer for 1 h at room temperature with gentle rocking (*see* Note 14).
9. During **step 8**, prepare SWB buffer containing [γ - 32 P]-ATP radiolabeled oligonucleotide (1.5–10 nM, 10⁶ cpm/mL), 0.25 % BSA, and 10 μ g/mL poly dI:dC (*see* Note 15).
10. Place blot from **step 8** in the solution prepared in **step 9** and incubate at 4 $^{\circ}$ C with gentle rocking overnight.
11. Wash blot with 50 mL of SWB buffer at least three times (*see* Note 16).
12. Air-dry washed blot, and then expose to film for autoradiography. A 1DGE-SWB is shown in Fig. 2a (*see* Note 17).

3.5 2-Dimensional Gel Electrophoresis SWB (2DGE-SWB)

1. Mix 25 μ L of protein sample (50–150 μ g NE) with 100 μ L IEF rehydration buffer, and distribute the sample mixture evenly across one well of the focusing tray using Bio-Rad PROTEIN IEF system (*see* Note 18).

10 % APS, and 3.35 mL of water for separating gel. Add to the gel cassette to 1.5 cm from the top and overlay with H₂O. The gel will polymerize in approximately 20 min, after which the H₂O layer is removed. For the upper (stacking) gel: 1.3 mL of 30 % acrylamide/BIS solution, 2.5 mL of 0.5 M Tris base pH 6.8, 10 μ L of TEMED, 50 μ L of APS, and 6.1 mL of H₂O. Add to the cassette containing the well comb and fill completely. The upper gel will require 30–60 min for polymerization.

¹²Load 10 μ L of 1 $\times$ Laemmli buffer into any unused wells to maintain even charge distribution.

¹³Protein renaturing is a crucial step in SWB assay. Some proteins may be difficult to renature from a denatured structure; consequently adjustment of the temperature and/or time may be needed for optimization.

¹⁴The blocking procedure chosen is dependent on subsequent experiments. For example, the blot can be blocked with 0.5 % polyvinyl pyrrolidone (PVP-40) in SW buffer; however, PVP-40 fragments cause complex spectra in a mass spectrometer. Nonetheless PVP-40 works best for renaturation and retention on the blot and gives the highest signal for SWB. Also, bovine serum albumin is not advised by most commercial mass spectrometry facilities as it may cause peak suppression in the proteins of interest. Alternatively, overnight blocking is recommended for better renaturing protein on the blot.

¹⁵Poly dI:dC is a commonly used competitor that reduces nonspecific interactions. Other competitors, such as mutant oligonucleotides or single-stranded DNA may also be applied to increase specific binding results.

¹⁶Efficient washing steps will result in increasing the signal-to-noise ratio as well as reduce nonspecific interactions.

¹⁷Exposure time can be adjusted to increase or decrease the strength of the signal.

¹⁸Enhanced rehydration buffer is used here. The standard rehydration buffer includes 8 M urea and eliminated thiourea.

2. Carefully remove the IPG strip (7 cm, linear, pH 3–10) protective cover with forceps starting from the acidic end (pH 3) and pulling towards the basic end (pH 10).
3. Place the gel side on the IPG strip down on top of the sample mixture with the acid end (pH 3) of the strip on the positive electrode in the tray. Overlay strip with mineral oil to prevent evaporation.
4. Perform active rehydration mode at 50 V for 12 h (*see* Note 19).
5. Place paper wicks at each end of strip between strip and electrode. Focus at 20 °C with 40,000 V · h.
6. Remove focused strip from tray and wash with rehydration buffer to remove mineral oil.
7. Place IPG strip in 15 mL of reduction buffer in a 15 mL conical tube. Incubate at room temperature with rocking for 15 min.
8. Transfer strip into another 15 mL conical tube containing 15 mL of alkylation buffer; incubate at room temperature in the dark with rocking for 15 min.
9. Rinse strip with 50 mL of water at room temperature, and then place onto a 12 % polyacrylamide hand-casting mini gel (*see* Note 20), as well as a protein ladder.
10. During **step 8**, heat 1 % agarose sealing solution, containing 0.001 % (w/v) of bromophenol blue and 125 mM Tris (pH 6.8), at 90 °C for 5 min to liquefy, and then cool to 50 °C before sealing. Apply agarose over the strip, carefully avoiding air bubbles.
11. Run SDS-PAGE at 100 V for 1.5 h or until the blue dye almost reaches the bottom of the gel.
12. Remove the gel from running chamber, and place into a clean plastic tray for blotting.
13. Repeat **steps 5–12** in Subheading 3.4. A 2DGE-SWB is shown in Fig. 2b; the results of probing a 2DGE-SWB with one probe, stripping [⁶], and reprobing with a different probe are shown in Fig. 3.

3.6 EMSA-Based 3D-SWB (3DGE-SWB)

1. Incubate 10 µL nuclear extract (50 µg) with radiolabeled 1.6 nM core promoter DNA or duplex oligonucleotide to form protein-DNA complex in EMSA buffer containing poly dI:dC (20 ng/µL) in a total volume of 25 µL at room temperature for 20 min and on ice for 10 min.
2. The 3 % or 6 % non-denaturing polyacrylamide gels for promoter complex or oligonucleotide complex, respectively, are pre-run at 4 °C for 30 min at 50 V.

¹⁹Passive rehydration is an alternative mode to rehydrate protein.

²⁰SDS-PAGE gel prepared with one long well. Two ends of IPG strip are cut off about half cm and inserted into the long well including a piece of filter paper containing absorbed 5 µL of protein ladder at one end. The top of strip is sealed with 1 % agarose sealing solution.

3. The protein mixture is applied to the gel for electrophoresis for 90 min at room temperature at 90 V (see Note 21).
4. The gel is electrotransferred to a PVDF membrane at 4 °C for 1.5 h in 20 % (v/v) methanol, 25 mM Tris base, and 192 mM glycine buffer at 100 mA.
5. After autoradiography, the DNA-protein complex is cut from the PVDF membrane at the position containing the specific complex using a clean, sharp scalpel. Then, the blotted band is cut into 1 × 1 mm pieces.
6. Place pieces into a 1.7 mL Eppendorf tube. Extract pieces twice with 1 mL of extraction buffer to soak the PVDF membrane and elute proteins at room temperature for 2 h each with gentle rocking. Combine the extracts.
7. Remove detergent from the extract using a Pierce Detergent Removal Spin column according to the manufacturer's protocol.
8. Concentrate protein sample to 20–25 µL using an Amicon Ultra 0.5 mL centrifugal concentrator (10,000 molecular mass cutoff) for IEF.
9. Repeat Subheading 3.5 for 2DGE-SWB. A 3DGE-SWB is shown in Fig. 4.

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²¹The percentage of acrylamide is dependent on the size of the DNA-protein complex to be separated. Optimal percentages range from 3 to 4 % for large complex, such as TF-core promoter DNA complexes, and 5–7 % for short oligonucleotide (20–25 bp) with NE.

Fig. 1. Flow chart of SWB experimental procedure. Protein samples are either prepared from nuclear extract or purified by other techniques. Oligonucleotides are generally 20 bp in size containing one response element or using core promoter DNA

Fig. 2.

1DGE-SWB and 2DGE-SWB results. Protein sample separated by an SDS-PAGE gel alone is called 1DGE-SWB. **(a)** A lane of a 12 % SDS-PAGE containing 1 μ g of HEK293 NE was electroblotted onto a PVDF membrane and probed with 10 nM of an oligonucleotide containing the response element bound by MEF2 (5'-TGGGCTATTTTATAGGG-3', annealed to its complement strand). **(b)** NE (50 μ g) was separated by IEF and 12 % SDS-PAGE and electroblotted to PVDF. It was then probed at 4 $^{\circ}$ C with 10 nM of the MEF2 oligonucleotide

Fig. 3.

Repeatedly probed SWB assay after enzymatic stripping. **(a)** 50 μ g HEK293 NE was separated by 2DGE. Proteins were electroblotted onto a PVDF membrane and SWB assay was performed as described in Subheading 3.5. The blot was first probed with 10 nM radiolabeled core c-jun promoter DNA (281 bp). **(b)** The blot was then stripped by alkaline phosphatase (2 U/mL) as described in [6] and re-probed with 10 nM of a radiolabeled oligonucleotide (5'-ACGCGAGCCAATGGGAAG-3, annealed with its complement) bound by CTF

Fig. 4.

3DGE-SWB using an AP1 oligonucleotide. (a) Fifty micrograms of HEK293 nuclear extract was mixed with 1.6 nM radiolabeled AP1 oligonucleotide (5'-CGCTTGATGACTCAGCCGGA-3' annealed with its complement), separated by 5 % non-denaturing PAGE, and electroblotted to PVDF. After autoradiography, the complex (C) band was excised and the proteins extracted. (b) The extract was then applied to 2DGE-SWB probed with 10 nM radiolabeled AP1 oligonucleotide. Panel b is reprinted from J. Chromatogr. A 1218, Jiang, D., Jia, Y., and Jarrett, H.W. "Transcription factor proteomics: Identification by a novel gel mobility shift-three-dimensional electrophoresis method coupled with southwestern blot and high-performance liquid chromatography-electrospray-mass spectrometry analysis", pages 7003–7015 (2011) with permission from Elsevier

Fig. 5.
A simple homemade desalting spin column. The column was prepared as described in Note 6