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Far western blotting as a rapid and efficient method for detecting interactions between DNA replication and DNA repair proteins

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Summary

Protein-protein interactions are required for the proper function of many biological pathways. Numerous biochemical and protein blotting methods are available for probing direct and indirect interactions between two protein binding partners. Here, we describe the methodology of far Western blotting, or immunodot blotting, as a technique for probing direct interactions between two proteins. We describe the utility of this approach as a rapid, qualitative screen for identifying novel protein binding partners. We also describe the importance of this technique for measuring differences in interaction between wild type and mutant forms of a known binding partner. Far Western blotting is a rapid and highly reproducible experimental approach for identifying and understanding the interaction between protein binding partners leading to new discoveries in the function and regulation of biological pathways.

Keywords

Far Western Blot; Immunodot Blot; Protein-Protein Interaction; Antibodies; Antigens

1. Introduction

Immunodetection of proteins separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) is an extremely powerful technique which has been common in the laboratory setting for several decades [1]. While this technique allows for the detection of a protein in a cell lysate, it lacks the capacity to inform the user of interactions that may have occurred between the protein of interest and other proteins in the cell. The denaturation of a protein during SDS-PAGE prevents the detection of interactions with proteins due to loss of secondary and tertiary structure. Conversely, native PAGE analysis can be very useful because it allows for the maintenance of protein structure and the detection of a protein within a complex. However, it has the limitations that some protein interactions are poorly detected by this technique and that large protein complexes are difficult to resolve in a

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⁷Membranes can be incubated with as little as 4 mL of protein binding buffer with prey protein but 6–7 mL seems to yield the best results. Incubation can also be done at room temperature for only 3 hours.

native gel [2]. Far Western blotting is an effective and efficient technique used to assay interactions that occur between natively, structured proteins. It can be used to specifically detect interactions between the protein of interest and any number of bait proteins immobilized on a solid support membrane, such as nitrocellulose. This is useful not only for the detection of interaction between full-length purified proteins, but also for identifying areas of interaction between purified proteins which may be truncated or have amino acid changes in putative binding interfaces [3, 4] (Figure 1). In addition to locating sites of potential interaction, this technique can provide a measure of relative binding if the concentrations of each protein being tested are known [5]. Far Western blotting can also be performed without having antibodies generated against your protein of interest through the use of functional affinity tags on the prey (capture) protein used in the assay. The protocol described here allows for the rapid screening and detection of potential protein-protein interactions and provides a method to qualitatively determine relative binding strength.

2. Materials

2.1 SDS Polyacrylamide Gel Components

1. Resolving Gel Buffer: 1.5 M Tris, pH 8.8. Add 100 mL water to a glass beaker. Add 181.81 g Tris Base to the beaker and add water to a total volume of 900 mL while mixing. Adjust pH to 8.8 with HCl, add water to a final volume of 1 L and filter sterilize.
2. Stacking gel buffer: 1.0 M Tris, pH 6.8. Add 100 mL ddH₂O to a glass beaker. Add 121.14 g Tris Base to the beaker. Then, add ddH₂O to a total volume of 900 mL while mixing. Adjust pH to 6.8 with HCl, add water to a final volume of 1 L and filter sterilize.
3. Acrylamide 40% solution. 19:1 Acrylamide : Bis-Acrylamide.
4. Ammonium persulfate: 10% (w/v) solution in ddH₂O. Prepare this solution fresh before making SDS-PAGE.
5. N, N, N, N'-tetramethyl-ethylenediamine (TEMED), stored at 4°C.
6. 5x SDS Electrophoresis Buffer: In a 1 L beaker, add ddH₂O to 100 mL. Add 15.1 g Tris base, 72.0 g glycine, and 5.0 g SDS. Fill to 1 L with ddH₂O. Dilute to 1x with ddH₂O for use in electrophoresis. [6]
7. 6X SDS sample buffer: add 7 mL stacking gel buffer (1.0 M Tris, pH 6.8) and 3 mL of glycerol to a 15 mL conical tube. Add 1 g SDS, 1 g bromophenol blue and 0.93 g DTT to the tube. Vortex the solution to mix and aliquot into 0.5 mL quantities and store at -70°C up to 6 months. [6]
8. Mini PROTEAN glass plates with 1 mm spacers and glass short plates.

2.2 Electrophoretic Transfer Components

1. Transfer Buffer, 10X: Add 30.3 g of Tris Base to 100 mL of ddH₂O in a 1 L beaker. Add 144.2 g of Glycine and fill to 1 L with ddH₂O. The solution is then filtered through a membrane with a 0.22 µm pore size. For use in electrophoresis,

add 100 mL of 10X transfer buffer to 700 mL of ddH₂O. Then add 200 mL of methanol to a total volume of 1 L. [6]

2. Nitrocellulose Membrane: Whatman Protran 0.22 μ M cut to an appropriate size.

2.3 Affinity Purification Components

1. Ponceau stain: in 1 mL of Glacial Acetic Acid dissolve 0.5 g Ponceau S. Add ddH₂O to a final volume of 100 mL [6].
2. Phosphate Buffered Saline, pH 7.6 (PBS): Mix 87 mL of 0.2 M Na₂HPO₄ and 13 mL of 0.2 M NaH₂PO₄. Check to ensure that the pH is 7.6. Add 8.766 g of NaCl. Fill to 1 L before filter sterilizing. Final concentration used is 20 mM phosphate, 150 mM NaCl.
3. Blocking Buffer: 5% w/v dried milk solids in PBS plus 0.05% v/v Tween-20.
4. Sodium Phosphate Buffer, pH 8.0: 94.7 mL of 0.2 M Na₂HPO₄, 5.3 mL 0.2 M NaH₂PO₄ filled to 1 L with ddH₂O. Final concentration used is 20 mM phosphate.
5. Strip Buffer: 5 mM glycine, 150 mM NaCl, pH 2.4

2.4 Immunoblotting Components

1. Blocking Buffer: 5% w/v dried milk solids in PBS plus 0.05% v/v Tween-20.
2. Protein Dilution Buffer: PBS plus 0.05% v/v Tween-20
3. Nitrocellulose Membranes: Whatman Protran 0.22 μ M cut to a usable size (about 1 cm² per dot). Alternatively, if a spot blot apparatus is used we recommend Bio-Rad Trans-Blot with a nitrocellulose membrane with a 0.45 μ M pore size.
4. Protein Binding Buffer: 5% w/v dried milk solids in PBS, 0.05% Tween-20, 0.2–0.4 μ M prey protein
5. Chemiluminescent Substrate: SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific #34080)

2.5 Antigens

1. Purified bait and prey proteins: purified to >95% purity as judged by SDS-PAGE.
2. Purified bovine serum albumin: 10 mg/mL (New England Biolabs) or other protein as an appropriate negative control.
3. Primary antiserum: Polyclonal antiserum is affinity purified and tested for cross reactivity with bait proteins. If primary antiserum shows no cross reactivity with other proteins, or is purchased monoclonal, affinity purification is unnecessary. Affinity purified antiserum is used after dilution in blocking buffer (often 1:2000). Antiserum generated by Covance is typically used in our laboratory as a primary antibody.
4. Secondary Antibody: Stabilized Peroxidase Conjugated Goat anti-Rabbit (Pierce / ThermoScientific #32460) used at a 1:2000 dilution in blocking buffer. If you are

using primary antibodies generated in mice or other animals, use the appropriate secondary antibody.

3. Methods

3.1 Affinity Purification of Antisera

1. Purified proteins are electrophoresed on a SDS-PAGE. Typically using 10 µg of purified protein per well and 10-100 µg total purified protein per gel. Note 1.
2. Transfer purified protein to a nitrocellulose membrane using electrophoretic transfer.
3. Stain proteins on the nitrocellulose membrane with Ponceau S, and rinse with ddH₂O to destain.
4. Cut the band of purified protein from the nitrocellulose membrane with a clean razor blade leaving as little extra space as possible surrounding the protein.
5. Block the membrane by adding blocking buffer and incubating at room temperature for twenty minutes with constant gentle shaking.
6. Wash the membrane twice for 2 minutes each time in PBS plus 0.05% Tween-20.
7. Incubate the strip of nitrocellulose binding your protein with 300 µL of antiserum for 1 hour with gentle mixing at room temperature. Note 2.
8. Following incubation, remove the antiserum. Note 3.
9. Wash the membrane twice for 15 minutes each in 1–2 mL of PBS.
10. Aliquot 100 µL of sodium phosphate buffer into three separate microcentrifuge tubes.
11. Strip the membrane by adding 300 µL of strip buffer to the membrane followed by incubation.
12. Incubate at room temperature for 30 seconds.
13. Remove the strip buffer from the membrane and then add the buffer to one of the microcentrifuge tubes containing sodium phosphate for neutralization of the solution.
14. Repeat steps 11–13 two more times.
15. Combine all three tubes of quenched strip buffer, which now contain the affinity-purified antibodies, in order to equalize the antibody titer.

SDS-PAGE can be performed with various well sizes depending on the amount of protein that is to be separated. If a large amount of purified protein is to be separated, it is more convenient to use much larger wells as opposed to multiple small wells since there will be less excess nitrocellulose to remove when the membrane is cut.

²The membrane can be cut in half prior to incubation with antisera and one half may be stored at –20°C if tightly wrapped in plastic. Additionally, when incubating with antiserum, it helps to incubate the piece of membrane in a microcentrifuge tube because this provides optimal surface to volume ratio for contact of the protein with the antiserum.

³The depleted antiserum may still contain a significant amount of antibodies and may be used for affinity purification again if the antiserum is in short supply.

16. Divide the affinity purified antibodies into new aliquots and store at 4°C.
17. Antibodies should be checked for titer by standard western blot methods following each purification prior to use since yields may vary between preparations. Note 4.

3.2 Immunodot Blotting

1. Serial dilute the bait proteins to be examined for interaction in protein dilution buffer to appropriate molarity for the experiment. Note 5.
2. Apply serial dilutions of protein samples to a precut, dry membrane leaving at least 1 cm of space between the centers of each dot of the series. Apply to the membrane either BSA or other protein which does not bind the prey protein as a negative control. Allow the membrane to dry before the next step. Note 6.
3. Place the membrane in blocking buffer for 1 hour at room temperature on an orbital shaker with gentle agitation, usually at approximately 20 RPMs, to block the membrane.
4. Remove blocking buffer and replace with protein binding buffer containing the prey protein for at least 6 hours of incubation at 4°C on an orbital shaker or rocker with gentle agitation. Note 7.
5. Following incubation with the prey protein, wash 3 times in blocking buffer for 5 minutes each with gentle rotation.
6. Incubate in blocking buffer containing affinity purified primary antiserum overnight at 4°C on an orbital shaker or rocker at approximately 20 RPMs. Alternatively, if the protein binding step was performed at 4°C overnight primary antiserum incubation can be performed at room temperature for 4–6 hours. Note 8.
7. Remove the primary antibody solution and wash 3 times with blocking buffer for 5 minutes each on an orbital shaker or rocker at low RPMs.
8. Place the blot in secondary antibody solution diluted 1:2000 in blocking buffer. Incubate on an orbital shaker or rocker at room temperature for 2–3 hours using low RPMs.
9. Following incubation with secondary antibody solution, wash once with blocking buffer for 5 minutes. Following the first wash, use two more wash steps with protein dilution buffer for 5 minutes each.

⁴Affinity purified antibodies are stored at 4°C in an effort to prevent loss of function of the antibodies due to multiple freeze thaw cycles. Sodium azide may also be added to 1 mM to prevent contamination.

⁵10 pmoles of monomeric bait protein seems to be a good starting point for detecting interactions. If interactions are hypothesized to be stronger, then the amount of protein used should be decreased to 2.5 pmoles or lower.

⁶Bio-Rad sells a dot-blot apparatus (Bio-Dot Apparatus), which allows for the exact spacing between protein samples (Bio-Rad #170-6545). When using this apparatus, we use a nitrocellulose membrane that has 0.45 µM pore size and the membrane is pre-wet in PBS prior to use. Rather than using the force of a vacuum, the protein is allowed to bind the membrane by gravity filtration. The vacuum is used only when placing the membrane onto and removing the membrane from the Bio-Dot apparatus.

⁸Our lab generally uses affinity purified primary antiserum diluted 1:250 in blocking buffer. It should be noted that the titer of affinity-purified antibody can vary between preparations and thus the dilution used may need to be adjusted for each experiment. We discard affinity purified primary antiserum after one use.

10. Activate the blot by addition of 1 mL of each component of the chemiluminescent substrate. Wash the substrate over the blot for 2–5 minutes and remove the blot from solution.
11. Expose blot to film for an appropriate length of time, depending on the strength of your signal. Note 9.
12. Following development of the film, examine each series. From the lowest molarity to the highest there should be an increase in signal intensity if the bait and prey proteins interact. The negative control protein should have no signal or signal increase since your antibodies should be specific to the prey protein, and your prey protein should not bind the negative control.

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References

1. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*. 1979; 76(9):4350–4354. [PubMed: 388439]
2. Wittig I, Schagger H. Advantages and limitations of clear-native PAGE. *Proteomics*. 2005; 5(17): 4338–4346. [PubMed: 16220535]
3. Simmons LA, et al. Beta clamp directs localization of mismatch repair in *Bacillus subtilis*. *Molecular cell*. 2008; 29(3):291–301. [PubMed: 18280235]
4. Klocko AD, et al. Mismatch repair causes the dynamic release of an essential DNA polymerase from the replication fork. *Molecular microbiology*. 2011; 82(3):648–663. [PubMed: 21958350]
5. Ohba T, et al. Dot far-western blot analysis of relative binding affinities of the Src homology 3 domains of Efs and its related proteins. *Analytical biochemistry*. 1998; 262(2):185–192. [PubMed: 9750131]
6. Gallagher, SR.; Wiley, EA. *Current protocols essential laboratory techniques*. Hoboken: John Wiley & Sons; 2008.

⁹A good starting point for exposure time with film is usually 1 minute, but often the exposure requires 5–10 minutes. If a satisfactory exposure time is not achieved before loss of the luminescent signal occurs, the blot can be washed and activated a second time by washing with protein dilution buffer for 1 minute. The blot can then be reactivated by repeating the application of chemiluminescent substrate. This seems to work best if the chemiluminescent substrate is applied in the dark room to prevent the early loss of signal.

A **B** **Figure 1. Detection of direct protein-protein interactions using immunodot blotting**

(A) Shown is a test for MutS binding partners. MutL, DnaN, and BSA were spotted in increasing amounts from 2.5 to 80 pmoles of monomer on a nitrocellulose membrane. The membrane was incubated with 100 nM purified MutS followed by incubation with the primary antibody, affinity purified anti-MutS at a 1:250 dilution. The membrane was subsequently incubated with the secondary antibody HRP-conjugated goat anti-rabbit at a 1:5000 dilution. (B) Shown is a test for MutL binding partners. Proteins DnaE, DnaN and BSA were spotted in increasing amounts from 2.5 to 80 pmoles of monomer as in (A). The

membrane was incubated with 400 nM purified MutL followed by a 1:250 dilution of the primary antibody, affinity purified anti-MutL. The membrane was subsequently incubated with a 1:2000 dilution of the secondary antibody HRP-conjugated goat anti rabbit.