

EFFICIENT ELECTROBLOTTING AND ON-MEMBRANE PROTEOLYSIS AT HIGH SENSITIVITY FOR SEQUENCE ANALYSIS

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The most effective final purification step for isolating proteins at low picomole levels prior to sequence analysis is to utilize either 1D or 2D polyacrylamide gel electrophoresis (PAGE). These separated proteins can then be either digested in the gel with a protease or the proteins can be electrotransferred onto a high retention PVDF membrane. One of the advantages of electroblotting proteins onto PVDF membranes is that replicate lanes from a single gel can be used for N-terminal sequencing, proteolysis/peptide separation/internal sequencing and/or other analysis methods such as Western analysis. In this tutorial, a high yield electroblotting method suitable for most proteins will be demonstrated and representative results will be illustrated. This method uses high retention PVDF membranes such as ProBlott or Trans-Blot, a 10 mM Tris, 100 mM Glycine, 10% methanol transfer buffer and a tank-type transfer unit with solid electrodes (1). The single most important factor in efficient electroblotting is the amount of SDS present in the gel during electrotransfer. High amounts of SDS interfere with protein binding to the PVDF membrane resulting in "blow through" while low amounts of SDS or too rapid dissociation of SDS from the protein during the transfer will dramatically reduce yields by leaving substantial amounts of the protein in the gel after transfer. Related factors that must be considered is the gel thickness (optimum is 1.0 - 1.5 mm), the amount of SDS in the gel matrix and in the electrode buffer, and the amount of methanol in the transfer buffer. Guidelines for identifying electrotransfer problems and ensuring optimal electrotransfer yields will be presented together with strategies for optimizing yields of "difficult" proteins including very high molecular weight proteins, membrane proteins and large peptides. In the second part of the tutorial, in situ proteolysis of electroblotted proteins using a modification (2) of the method described by Fernandez et al. (3) will be described and key steps will be demonstrated. This method can be readily optimized and routinely used to obtain internal sequence information when as little as 20 picomoles of the desired protein is applied to an SDS gel, which typically corresponds to about 12 to 16 picomoles on the PVDF membrane. More rigorous optimization of each step of the method including the HPLC separation can lead to useful internal sequence information when as little as 5 - 10 picomoles of the target protein is applied to the gel (3 - 8 picomoles on the membrane).

1. J. Mozdzanowski and D.W. Speicher. 1992. Analytical Biochemistry 207:11 -18.
2. S. Best, D.F. Reim, J. Mozdzanowski, and D. W. Speicher, 1994. Techniques in Protein Chemistry V, pp. 565-574.
3. J. Fernandez, M. DeMott, D. Atherton, and S.M. Mische. 1992. Analytical, Biochemistry 201: 255-264.

OPTIMIZING ELECTROBLOTTING AND IN SITU DIGESTIONS ON PVDF

- Selecting the proper gel
- Sample preparation
- Gel electrophoresis separation
- Electrotransfer
- Staining & storage of the membrane
- Protease digestion
- HPLC purification

OPTIMIZING ELECTROBLOTTING

Where did my protein go???

- Expected recovery = 40 - 80%
- Possible recovery = 0 - 80%

Myths

- You can over-transfer!
- Large proteins can not be transferred well! -Thin gels are best!
- All PVDF membranes are the same!
- No need to save the gel after transfer!

SELECTION/PREPARATION OF THE PROPER GEL

- Protein Rf should be between 0.3 and 0.7
- Only 1.0 to 1.5 mm thick gels!
- 0.2% SDS in gel and electrode buffer
- Full sized gel is best
- Laemmli gel system preferred
- High purity reagents only - Bio-Rad, etc
- Filter all gel solutions before use
- Minimize gel solution storage
- Store total gel with stacker at r.t. 24hr.

SAMPLE PREPARATION

- Avoid amine reactive reagents (urea, etc.)
- Use high purity sucrose in solubilization buffer
- Use reducing reagent, if possible
- Heat at 37 °C for 5 -15 min, NOT 90 °C

GEL ELECTROPHORESIS

- Electrode buffer SDS conc is important (0.2%)
- Upper buffer volume is important
- final SDS conc varies with gel % due to differing run times - do not run tracking dye off gel!
- Add 0.1 mM thioglycolate to upper buffer
- **Include standards at known conc.!
- **Include expt. controls!

PRE-TRANSFER

- Pre-soak gel in transfer buffer for 2-5 min when using Trans-Blot or ProBlott
- Pre-wet PVDF in methanol, then transfer buffer
- Remove air bubbles from membrane
- **Avoid air bubbles between gel/PVDF/ filters
- **Do not let PVDF dehydrate during setup

TRANSFER CONDITIONS

- **Use tank-type transfer unit
- ** Use solid plate electrodes (Bio-Rad)
- **Gel/PVDF/+electrode
- 1 gel per transfer unit
- 10 mM Tris, 100 mM glycine, 10% methanol
- 200 - 250 mA for 2 to 4 hrs.

STAINING & STORAGE

- **Rinse thoroughly with Milli-Q water
- Preferred stain is Amido Black> Ponceau S
- Destain with Milli-Q water
- Dry in dust-free location
- Seal immediately in ziplock bag
- Store -20°C

AMIDO BLACK 10B STAIN (NAPHTHOL BLUE-BLACK)

1. 1% Amido black (Sigina) in 10% HOAc
 2. Stain 1 min.
 3. Destain w/ 5% acetic acid - 1 min.
 4. Wash 3X w/ Milli-Q water, 5 min each.
- Dry in dust-free location.

TROUBLESHOOTING ELECTROTRANSFER

No or little protein on blot

- Did standard proteins transfer?
- Stain transferred gel w/ Coomassie blue.
- Stain duplicate lane w/out transfer.
- Electrodes reversed at power supply or unit?
- Sandwich inserted backwards?
- PVDF was not wetted w/methanol?
- Adjust % methanol and/or gel%.

Unstained areas on blot

- Round spots = air bubbles
- Irregular spots = PVDF "de-solvated" during assembly.
- Streaks or smears on gel
- Sandwich was not tight, gap between gel and membrane.
- Fingerprints or blotches on PVDF
- Wear latex gloves rinsed w/ Milli-Q, then methanol
- Handle PVDF only by ends with gloves or clean forceps

Protein left in gel & standards are on membrane

- Eliminate methanol, eliminate gel presoak.
- Decrease gel %, especially if $R_f < 0.5$.
- Try 0.005% SDS 'in transfer buffer.
- Protein "blew through" membrane & standards are on membrane
- increase methanol to 20%.
- increase gel pre-soak volume & time.
- increase gel %, especially if $R_f > 0.5$.

SAMPLE PREP - IN SITU DIGESTION

1. Clean tubes w/ 0.1% TFA, 50% ACN.
 2. Excise bands w/ clean tweezers & scalpel.
 3. **Use <100 mm² membrane per tube.
 4. Pre-wet PVDF w/ methanol
 5. Destain w/ 0.2 mM NaOH, 20% ACN
 6. Rinse 5X w/ Milli-Q water.
- Note: Sonicate samples during washes

IN SITU DIGESTION

Modified trypsin (Promega) is preferred

7. Add 50 μ L digestion buffer per tube to wet PVDF (100 mM Tris, 1% Triton, 10% ACN, pH 8.0)
8. Add 2 μ l trypsin at 0.1 μ g/ μ l
9. Incubate 37°C for 4 hr.
10. Add another 2 μ L trypsin
11. Digest overnight at 37°C.

Sample collection, storage, separation

12. Sonicate sample 5 min, centrifuge
13. Remove supernatant, add 3.7 μ l 5% TFA.
14. Extract membrane w/ 25 μ l digestion buffer.
15. Extract membrane w/ 25 μ l 0. 1% TFA.
16. Store pooled extracts at -20'C
17. Separate on Zorbax 300 SB-C18 column (2.1 X 150 mm)

IN SITU DIGESTION - Helpful Hints

1. If PVDF surface area >100 mm², splice into separate tubes. Combine before HPLC.
2. Triton RTX-100 is a 10% solution, not neat.
3. Always digest a protein standard in parallel with samples (60 pmoles HSA).
4. Use replicate aliquots of a large scale protein digest to Q.C. HPLC system (5011000 pmoles).
5. Measure delay time from UV to collector directly.
6. Drop size for peak collection (2.1 mm column) should be <10 μ l.

Related References

Gillespie, R.G. and Gillespie, S.K.H. (1997) Anal. Biochem. **246**, 239-245.
Improved Electrophoresis and Transfer of Picogram Amounts of Protein with Hemoglobin.