Practical Technique of Western Blotting

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Abstract: Western blotting is a widely used in protein detection method. This article describes the principle theory and technique for Western blotting procedure. [The Journal of American Science. 2008;4(2):1-3]. (ISSN 1545-1003).

Keywords: protein; SDS-polyacrylamide gel electrophoresis (SDS-PAGE); Western blotting

1. Introduction

Western blotting is powerful technique that is widely used in the protein detection. Since 1979, protein blotting has evolved greatly (Kurien, 2006). Western blotting analysis can detect the protein in a solution and give the protein information sensitively (Dechend, 2006; Ma, 1994; 2004; Peter-Katalinic, 2005; Sakudo, 2006; Westermeier, 2005). As the general process: 1. Separate the proteins using SDS-polyacrylamide gel electrophoresis (SDS-PAGE); 2. Transfer the protein from SDS-gel to a nitrocellulose membrane (electric transfer); 3. Put the primary antibody on the membrane; 4. Use the secondary antibody (this antibody should be an antibody-enzyme conjugate, e.g., horseradish peroxidase (HRP)); 5. Use the dye and read the result. As an application, Western blotting method revealed a significant decrease in endothelin-A receptor protein in left circumflex coronary arteries (Knudson, 2006). This article describes the principle theory and technique for Western blotting method.

I. Tissue Sample Preparation:

1. Isolate tissue (about 1 gram).
2. Put tissue in 3 volume of extract buffer.
3. Extract buffer (Table 1): The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes at 8.0 pH. PMSF is usually stored as a 10 mM or 100 mM stack solution (1.74 or 17.4 mg/ml in isopropanol) at –20^{o}C.
4. Homogenize sample under ice.
5. Centrifuge sample at 10,000 rpm for 10 minutes at 4^{o}C.
6. Keep supernatant at -70^{o}C until usage.

II. SDS PAGE:

1. Use 12% SDS gel. 12% SDS gel preparation is shown in Table 2 and an optional 12% SDS gel preparation reagent amount is shown in Table 3.
2. Take 50 ul of sample and add an equal volume of 2 x SDS gel-loading buffer. 2 x SDS gel-loading buffer is shown in Table 4. 2 x SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock (Dissolve 3.09 g of dithiothreitol in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterilize by filtration. Dispense into 1-ml aliquots and store at –20^{o}C).
3. Boil the sample (in loading buffer) at 100^{o}C for 3 – 5 minutes.
4. Load the sample for electrophoresis: 8 V/cm (6 x 8 = 48 volts) before the bromophenol blue (dye) front has moved into the resolving gel and 15 V/cm (6 x 15 = 90 volts) until the bromophenol blue reaches the bottom of the resolving gel.
5. Make the gel for transfer in transfer buffer: 0.65 mA/cm2 (about 100 volts) for 1.5 – 2 hours, or 30 volts overnight, on ice.
6. Western blotting transfer buffer (Table 5).
7. Block the filter with blocking buffer for 1 – 2 hours at room temperature (0.1 ml blocking solution per cm^{2} filter), with gentle agitation on a platform shaker. Blocking solution is shown in Table 6 and Phosphate-buffered saline (PBS) (pH 7.4, 1000 ml) is shown in Table 7.

8. Discard blocking solution and immediately incubate filter with primary antibody.
9. Add 10 ml (0.1 ml of blocking solution per cm^2 of filter). Blocking solution is shown in Table 8.
10. Add 0.005 ml of primary antibody (1:2000) in to blocking solution.
11. Incubate at 4°C for 2 hours or overnight with gentle agitation on a platform shaker.
12. Discard blocking solution and wash filter 3 times (10 minutes each time) with 250 ml of PBS.
13. Incubate the filter with 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) (phosphate-free, azide-free blocking solution) for 3 times for 10 minutes each time.
14. Immediately incubate the filter with secondary antibody.
15. Add 10 ml of phosphate-free, azide-free solution (150 mM NaCl, 50 mM Tris-HCl, 5% nonfat dry milk pH 7.5). Phosphate-free, azide-free blocking solution (pH 7.5, 1000 ml) is shown in Table 9.
16. Add 0.005 ml of secondary antibody solution (1:2000).
17. Incubate 1 – 2 hours at room temperature with gentle agitation.
18. Discard secondary and wash with 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) (phosphate-free, azide-free solution) for 3 times for 10 minutes each time.

III. Alkaline phosphatase stain:

1. Add 5 ml of the substrate 5-brono-4chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma).
2. Observe the filter for the blue color on the filter (about 20 minutes).
3. Discard BCIP/NBT solution when the bands are clear (about 20 minutes).
4. Immediately stop the enzymatic reaction by add water.
5. Cover the filter with plastic membrane and keep the filter.
6. Analyze the blue bands and compare the color.

The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes, at 8.0 pH. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at –20°C.

1X SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock (Dissolve 3.09 g of dithiothreitol in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterilize by filtration. Dispense into 1-ml aliquots and store at –20°C).

IV. Overall of Western blotting solutions

Tissue Extract buffer, 100 ml
50 mM Tris-HCl (pH 8.0), 0.6 g (Or 50 mM HEPES (pH 7.0), 1.19 g)
150 mM NaCl, 0.88 g
0.02% sodium azide, 0.02 g
0.1% SDS, 0.1 g
0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.01 g
0.001 mg/ml aprotinin, 0.1 mg
1% Nonidet P-40 (NP-40), 1 ml (Or 1% Triton X-100, 1 ml)
2 X SDS gel-loading buffer, 100 ml
100 mM Tris-HCl (pH 6.8), 1.21 g
200 mM dithiothreitol
4% SDS, 0.4 g
0.2% bromophenol blue, 0.2 g
20% glycerol, 20 ml
SDS 5x Running Buffer, pH 8.3, 1000 ml
Tris, 125 mM, 15.14 g
Glycine, 1.25 M, 93.84 g
SDS, 0.50%, 5 g

Western Transfer Buffer, 1000 ml
Tris, 48 mM, 5.814 g
Glycine, 39 mM, 2.928 g
SDS, 0.04%, 0.37 g, 3.7 ml of 10% SDS
Methanol, 20%, 200 ml
Blocking solution, 100 ml, in 100 ml phosphate-buffered saline (PBS, pH 7.4)
Nonfat dried milk, 5%, 5 g
Antifoam A, 0.01%, 10 ml
Sodium azide, 0.02%, 20 mg
Phosphate-buffered saline (PBS), pH 7.4, 1000 ml (adjust to pH 7.4 with HCl)
NaCl, 8 g
KCl, 0.2 g
Na_2HPO_4, 1.44 g
KH_2PO_4, 0.24 g
Phosphate-free, azide-free blocking solution, 1000 ml (adjust pH with 12 N HCl about 3.35 ml)
150 mM NaCl, 8.766 g
50 mM Tris-HCl (pH 7.5), 6.057 g
5% (w/v) nonfat dried milk

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