Introduction of Western Blotting (WB) and Enzyme-linked Immunosorbent Assay ELISA

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Abstract: Western blotting (WB) and enzyme-linked immunosorbent assay (ELISA) are the two most useful and sensitive methods to measure the ng/ml to pg/ml ordered materials in the solution, such as in tissue, serum, urine and cultured cells, etc, and they are especially widely used in protein detection. This article describes the principle theories and techniques for WB and ELISA procedure.

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1. Introduction

Western blotting (WB) and enzyme-linked immunosorbent assay (ELISA) are widely used in the protein detection (Savige, 1998). The name of Western blotting is also called Western blot, and the two are a same thing. ELISA is the abbreviation of enzyme-linked immunosorbent assay (Ma and Shieh, 2006).

Since the inception of the protocol for protein transfer from an electrophoresed gel to a membrane by Towbin in 1979 (Towbin, 1979), protein blotting has evolved greatly (Kurien, 2006). Western blotting analysis can detect one protein in a solution that contains any number of proteins and giving the protein information (Dechend, 2006; Ma, 1994; 2004; Peter-Katalinic, 2005; Sakudo, 2006; Westermeier, 2005). Western blotting method is normally used with a high-quality antibody directed against a desired protein. First, preparation of the sample such as homogenizing tissues and centrifuge it to get the protein supernatant sample. Secondly, separating the proteins using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (SDS is the abbreviation of sodium dodecyl sulfate, synonymously sodium lauryl sulfate) is done, and this separates the proteins by protein molecular size. Third, transfer the protein from SDS-gel to a nitrocellulose membrane or polyvinylidene difluoride (PVDF) membrane (electric transfer) (PVDF Transfer Membranes are made of high-quality polyvinylidene difluoride and provide high binding capacity for proteins and nucleic acids for Western, Southern, and Northern blotting methods). Fourth, block the membrane's non-specific sites by 5% non-fat milk (or bovine serum albumin, also known as BSA or Fraction V). Fifth, put the primary antibody on the membrane. Sixth, use the secondary antibody (this antibody should be an antibody-enzyme conjugate, e.g., horseradish peroxidase (HRP). Finally, use the dye and read the result (expose to the film or scan/read the dyed membrane directly by a digit blot scanner. Western blotting and ELISA are very efficient in the protein measurement (Knudson, 2006). The basic principle of an ELISA is to use an enzyme to detect the binding of antigen (Ag) and antibody (Ab). The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Ags or Abs in a sample, depending on how the test is designed. However, if a protein is degraded quickly, Western blotting and ELISA won't detect it well. In this case, radio-immune precipitation can be used for the protein detection.

2. Western Blotting

Western blotting is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose or PVDF membrane) and combine with antibodies specific to the protein. The secondary antibody can be stained and pictured by a film. The film with the protein binds can be kept for a long time and scanned any time it needs to quantity the protein levels. As a result, researchers can

examine the amount of protein in a given sample and compare levels between several groups. Other techniques also using antibodies allow detection of proteins in tissues (immunohistochemistry) and cells (immunocytochemistry).

The name Western blotting is a pun on the name Southern blotting, a technique for DNA detection and the detection of RNA is termed northern blotting.

2.1 Brief descriptions of the steps in a Western blotting

2.1.1 Tissue preparation

Typically, samples are taken from either tissue or from cell culture. The samples are cooled or frozen rapidly. They are homogenized using sonication or mechanical force. The resulting "whole-cell homogenate" or "whole-cell fraction" can be used as is, or subjected to centrifugation in a series of steps to isolate cytosolic (cell interior) and nuclear fractions. The prepared sample is then assayed for protein content so that a consistent amount of protein can be taken from each different sample.

Samples are boiled from one to five minutes in a buffer solution (e.g. Laemmli's buffer), containing dye, a sulfurous compound - typically beta-mercaptoethanol, and a detergent known as sodium dodecyl sulfate, or SDS. The boiling denatures the proteins, unfolding them completely. The SDS then surrounds the protein with a negative charge and the beta-mercaptoethanol prevents the reformation of disulfide bonds.

2.1.2 Gel electrophoresis

The proteins of the sample are separated according to molecular weight using gel electrophoresis. Gels have various formulations depending on the lab, molecular weight of the proteins of interest. Polyacrylamide gels are most common. Since the proteins travel only in one dimension along the gel, samples are loaded side-by-side into wells formed in the gel. Proteins are separated by mass into bands within each lane formed under the wells. One lane is reserved for a marker, or ladder, a commercially available mixture of proteins having defined molecular weights. Buffers and gels can be prepared by the researchers by bought from a company such as Bio-Rad.

It is also possible to use a 2-D gel (two dimensions) which spreads the proteins from a single sample out in two dimensions and proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

2.1.3 Transfer

The polyacrylamide gel is good for separating of protein, but not suitable for the staining and the further detecting. In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or PVDF. The membrane is placed face-to-face with the gel, and current is applied to large plates on either side. The charged proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.

2.1.4 Blocking

Since the membrane has been chosen for its ability to bind protein, steps must be taken to prevent non-specific protein interactions between it and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically bovine serum albumin (BSA) or non-fat dry milk, with a minute percentage of detergent such as Tween 20 or colloidal carbon.

2.1.5 Detection

During the detection process the membrane is probed for the protein of interest with antibodies, and links them to a reporter enzyme, which drives a colorimetric or photometric signal. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

2.1.5.1 Two step

Primary Antibody

Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest. This is the primary antibody. After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/ml) is incubated with the membrane under gentle agitation. Typically, the solution is comprised of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding

(specific and non-specific).

Secondary Antibody

After rinsing the membrane to remove unbound primary antibody, it is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing enhanced signal. Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane. A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like *Staphylococcus* Protein A with a radioactive isotope of iodine. Since non-rediocticity methods are safer, quicker and cheaper, there are few groups to use the radioactive label method now .

2.1.5.2 One step

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps. It is possible to combine the detectable label to any primary antibody, but it costs more. This is suitable for that the antibody are needed in a big amount in the market.

2.1.6 Analysis

After the unbound probes are washed away, the Western blotting is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

2.1.6.1 Colorimetric detection

The colorimetric detection method depends on incubation of the Western blotting with a substrate that reacts with the reporter enzyme (such as alkaline phosphatase or horseradish peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different colour that precipitates next to the enzyme and thereby stains the nitrocellulose membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

2.1.6.2 Chemiluminescence

Chemiluminescent detection methods depend on incubation of the Western blotting with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which captures a digital image of the Western blotting. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used. The new reagent, enhanced chemiluminescent (ECL) detection is considered to be among the most sensitive detection methods for blotting analysis.

2.1.6.3 Radioactive detection

Radioactive method is more sensitive. Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest. The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high and ECL provides a useful alternative.

2.1.6.4 Fluorescent detection

The fluorescently labeled probe is excited by

light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped which appropriate emission filters which captures a digital image of the Western blotting and allows further data analysis such molecular weight analysis and a quantitative Western blotting analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

2.1.7 Secondary probing

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 100% methanol or isopropanol before using. PVDF membranes also tend to be thicker and much more resistant to damage incurred by normal manipulation.

2.2 Medical diagnostic applications

The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody. A Western blotting is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease'). Some forms of Lyme disease testing employ Western blotting (http://en.wikipedia.org/wiki/Western_blot).

2.3 **Tissue Sample Preparation**

2.3.1 Isolate tissue (about 0.2 gram).

2.3.2 Put tissue in 5 volume of extract buffer (weight/weight).

2.3.3 Extract buffer (Table 1): The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes at 8.0 pH buffer. PMSF is usually stored as a 10 mM or 100 mM stack solution (1.74 or 17.4 mg/ml in isopropanol) at –20°C.

2.3.4 Homogenize sample under ice.

2.3.5 Centrifuge sample at 10,000 rpm for 20 minutes at 4°C, and collect the supernatant that contains the target protein for the measurement.

2.3.6 Aliquot the supernatant sample (30-100 ul each tube).

2.3.7 Keep supernatant at -70°C until usage.

2.4 Tissue Sample Preparation

2.4.1 Isolate tissue (about 0.2 gram).

2.4.2 Put tissue in 5 volume of extract buffer (weight/weight).

2.4.3 Extract buffer (Table 1): The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes at 8.0 pH buffer. PMSF is usually stored as a 10 mM or 100 mM stack solution (1.74 or 17.4 mg/ml in isopropanol) at –20°C.

2.4.4 Homogenize sample under ice.

2.4.5 Centrifuge sample at 10,000 rpm for 20 minutes at 4°C, and collect the supernatant that contains the target protein for the measurement.

2.4.6 Aliquot the supernatant sample (30-100 ul each tube).

2.4.7 Keep supernatant at -70°C until usage.

2.5 SDS-PAGE [sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis]

2.5.1 Use 4-12% SDS gel (Novex 4-12% Tris-Glycine Gel, 1.0 mm X 10 well, or, Acalogue No. EC6035BOX, Invitrogen). Or, the 12% SDS gel preparation can be prepared as shown in Table 2 and an optional 12% SDS gel preparation reagent amount is shown in Table 3.

2.5.2 Take 20 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 reduce reagent can be stored at room temperature. Reduce reagent could be beta-mercaptoethanol or dithiothreitol. Beta-mercaptoethanol or dithiothreitol should be added in the gel-loading buffer just before using. For beta-mercaptoethanol: 380 ul of gel-loading buffer plus 20 ul of beta-mercaptoethanol (loading buffer should be put at room temperature about 1-2 hours. For dithiothreitol, it should then be added just before the buffer is used also, 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).

2.5.3 Boil the sample (in loading buffer) at 95°C

for 1 minute.

2.5.4 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.

2.6 Electronic transfer

2.6.1 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.

2.6.2 Western blotting transfer buffer (Table 5).

2.7 Immunological analysis

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.

2.7.1 Discard blocking solution and immediately incubate filter with primary antibody.

2.7.2 Add 10 ml (0.1 ml of blocking solution per cm^2 of filter). Blocking solution is shown in Table 8.

2.7.3 Add 0.005 ml of primary antibody (1:2000) in to blocking solution.

2.7.4 Incubate at 4°C for 2 hours or overnight with gentle agitation on a platform shaker.

2.7.5 Discard blocking solution and wash filter 3 times (10 minutes each time) with 250 ml of PBS.

2.7.6 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.

2.7.7 Immediately incubate the filter with secondary antibody.

2.7.8 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.

2.7.9 Add 0.005 ml of secondary antibody solution (1:2000).

2.7.10 Incubate 1 – 2 hours at room temperature with gentle agitation.

2.7.11 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.

2.8 Alkaline phosphatase stain

2.8.1 Add 5 ml of the substrate 5-brono-4chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma).

2.8.2 Observe the filter for the blue color on the filter (about 20 minutes).

2.8.3 Discard BCIP/NBT solution when the bands are clear (about 20 minutes).

2.8.4 Immediately stop the enzymatic reaction by add water.

2.8.5 Cover the filter with plastic membrane and keep the filter. 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).

Table 1. Extract buffer for Western blotting

50 mM Tris-HCl (pH 8.0) or 50 mM HEPES (pH 7.0)
150 mM NaCl
0.02% sodium azide
0.1% SDS
0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF)
0.001 mg/ml aprotinin
1% Nonidet P-40 (NP-40) or 1% Triton X-100.

Table 2. 12% SDS gel preparation reagent amount (l)

	Separating gel (12%)	Stacking gel (4%)
Water	1672	3020

Tris-HCl	1250 (1.5 M, pH 8.8)	1250 (0.5 M, pH 6.8)
SDS (10%)	50	50
Acr-Bis (30%)	2000	650
AP	25	25
TEMED	3	5
Sum	5000	5000

(AP is ammonium persulfate)

Table 3. Optional 12% SDS gel preparation reagent amount (l)

	Separating gel (%12)	Stacking gel (4%)
Water	3344	3020
Tris-HCl	2500 (1.5 M, pH 8.8)	1250 (0.5 M, pH 6.8)
SDS (10%)	100	50
Acr-Bis (30%)	4000	650
AP	50	25
TEMED	6	5
Sum	10000	5000

(AP is Ammonium persulfate)

Table 4. 2 x SDS gel-loading buffer for Western blotting, 100 ml

62.5 mM Tris-HCl (pH 6.8), (Tris MW = 121.1, Sigma Catalog T-1503)
200 mM dithiothreitol
2% SDS (SDS MW = 288.38, Bio-Rad Catalog 161-0301)
0.01% bromophenol blue
0.25% glycerol

Table 5. 10 x SDS Running Buffer, pH 8.3, 1000 ml

Tris, 250 mM, 30.275 g (Tris MW = 121.1, Sigma Catalog T-1503)
Glycine, 1.92 M, 144.13 g (Glycine MW = 75.07, Bio-Rad Catalog 161-0717)
SDS, 1%, 10 g (SDS MW = 288.38, Bio-Rad Catalog 161-0301)

Table 6. Western blotting transfer buffer (1000 ml)

Chemicals	Concentration	Amount	M.W.
Tris	25 mM	3.03 g	121.1
Glycine	192 mM	14.41 g	75.07
Methanol	20%	200 ml	

Table 7. Phosphate-buffered saline (PBS), pH 7.4, 1000 ml (adjust to pH 7.4 with HCl)

NaCl, 150 mM, 8.77 g, MW = 58.44, Sigma Catalog S-9888
KCl, 2.7 mM, 0.2 g, MW = 74.55
Na_2HPO_4, 10.1 mM, 1.44 g, MW = 142.0
KH_2PO_4, 1.8 mM, 0.24 g, MW = 136.09

Table 8. 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

0.2 ml Tween 20

Sodium azide: 1 ml of 2% solution

Table 9. Blocking solution

Blocking solution, 10 ml, in PBS (pH 7.4)	
Nonfat dried milk	5%
Antifoam A	0.01%
Sodium azide	0.02%

Table 10. Phosphate-free, azide-free blocking solution (pH 7.5, 1000 ml)

NaCl	150 mM	8.766 g
Tris-HCl (pH 7.5)	50 mM	6.057 g
12 N HCl		about 3.35 ml
Nonfat dried milk	5% (w/v)	

Overall of Western blotting solutions (Table 11)

Table 11. Overall table 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) (Tris 1.21 g)
200 mM dithiothreitol
4% SDS, 0.4 g
0.2% bromophenol blue, 0.2 g
20% glycerol, 20 ml
1.5 M Tris-HCl, pH 8.8, 300 ml
Tris, 54.5 g
HCl, 12 N, 6.375 ml
0.5 M Tris-HCl, pH 6.8, 300 ml
Tris, 18.17 g
HCl, 12 N, 11.5 ml
10 x SDS-PAGE Running Buffer, pH 8.3, 1000 ml
Tris, 250 mM, 30.275 g (Tris MW = 121.1, Sigma Catalog T-1503)
Glycine, 1.92 M, 144.13 g (Glycine MW = 75.07, Bio-Rad Catalog 161-0717)
SDS, 1%, 10 g (SDS MW = 288.38, Bio-Rad Catalog 161-0301)
Western Blotting Transfer Buffer, 1000 ml, keep at 4^{o}C before using
Tris, 25 mM, 3.03 g, MW = 121.1
Glycine, 192 mM, 14.41 g, MW = 75.07
Methanol, 20%, 200 ml

Phosphate-buffered saline (PBS), pH 7.4, 1000 ml (adjust to pH 7.4 with HCl)
NaCl, 150 mM, 8.77 g, MW = 58.44, Sigma Catalog S-9888
KCl, 2.7 mM, 0.2 g, MW = 74.55
Na_2HPO_4, 10.1 mM, 1.44 g, MW = 142.0
KH_2PO_4, 1.8 mM, 0.24 g, MW = 136.09
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
0.2 ml Tween 20
Phosphate-free, azide-free blocking solution, 1000 m l (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
Transfer Buffer (Optional), 1000 ml
20 mM Tris-HCl, pH 8.0, 2.42 g, Tris MW = 121.1
150 mM Glycine, 11.26 g, Glycine MW = 75.07
20% methanol, 200 ml
TBS (Optional), 1000 ml
125 mM NaCl, 7.3 g, NaCl MW = 58.44, Sigma Catalog S-9888
25 mM Tris pH 8.0, 3 g, Tris MW = 121.1

2.9 More description of Western blotting

2.9.1 SDS-PAGE

SDS-PAGE is the abbreviation of sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis.

Agarose gels are best for isolating larger molecules (such as DNA), and SDS-PAGE is the best choice to isolate smaller molecules (such as proteins).

The common usages of SDS-PAGE could be: (1) Determining protein size; (2) Identifying protein sort; (3) Detecting protein sample purity; (4) Finding disulfide bonds in proteins; (5) Quantifying proteins; (6) Blotting applications.

The SDS portion is a detergent. The SDS detergent makes the protein from its native shape to a denatured form. The denatured protein is a linear form that can be run by the gel depending on its molecule size.

SDS is an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform charge, so that they can be separated according to the molecule weight of the proteins. The SDS has a hydrophobic tail that interacts strongly with protein (polypeptide) chains. The number of SDS molecules that bind to a protein is proportional to the number of amino acids that make up the protein. Each SDS molecule contributes two negative charges, overwhelming any charge the protein may have. So that, in this case the negative charges that the proteins have will be linearly related to their amino acid numbers. Averaged, a protein molecular weight is linearly related to its amino acid numbers (one amino acid is 110 daltons averagely). SDS also disrupts the forces that contribute to protein folding (tertiary structure), ensuring that the protein is not only uniformly negatively charged, but linear as well.

The polyacrylamide gel electrophoresis works in a similar fashion to an agarose gel, separating protein molecules according to their size. In electrophoresis, an electric current is used to move the protein molecules across a polyacrylamide gel. The polyacrylamide gel is a cross-linked matrix that functions as a sort of sieve to help catching the molecules as they are transported by the electric current. The polyacrylamide gel acts somewhat like a three-dimensional mesh or screen. The negatively charged protein molecules are pulled to the positive end by the current, but they encounter resistance from this polyacrylamide mesh. The smaller molecules are able to navigate the mesh faster than the larger one, so they make it further down the gel than the larger

molecules. This is how SDS-PAGE separates different protein molecules according to their size.

Once an SDS-PAGE gel is run, we need to fix the proteins in the gel so they don't come out when you stain the gel. Acetic acid 25% in water is a good fixative, as it keeps the proteins denatured. The gel is typically stained with Coomasie blue dye R250, and the fixative and dye can be prepared in the same solution using methanol as a solvent. The gel is then destained and dried (Ji, 2006).

2.9.2 Immunoassay

Transferring proteins to membranes from gels lets the proteins be more stable adhere on the membrane to be efficiently detected with various probes. Polyacrylamide is really good to separate proteins, but not suitable for the further analysis. To transfer the proteins to a stable membrane is useful for the further analysis. The most popular type of probe of immobilised proteins is an antibody. Chemiluminescent substrates have begun to be used because of their greater detection sensitivity. Other possibilities for probing include the use of fluorescent or radioisotope labels (fluorescein, ^{125}I). Probes for the detection of antibody binding can be conjugated anti-immunoglobulins; conjugated staphylococcal Protein A or probes to biotinylated / digoxigeninylated primary antibodies.

The immunoassay is normally done by blocking the transfer membrane with a concentrated protein solution (10% foetal calf serum or 5% non-fat milk powder) to prevent further non-specific binding of proteins, then incubating the membrane in a diluted antiserum/antibody solution, washing the membrane, incubating the membrane in diluted conjugated probe antibody or other detecting reagent, further washing, and the colorimetric / autoradiographic / chemiluminescent detection.

The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass. Proteins are first separated by mass in the SDS-PAGE, then specifically detected in the immunoassay step.

It is also possible to use a similar technique to elute specific antibodies from specific proteins resolved out of a complex mixture, many of whose components react with a given antiserum: one can electrophorese a mixture of proteins, cut out a specific band from a gel or membrane, and use this to fish out specific antibodies from a serum.

Staining of proteins in gels may be done using the standard Coomassie brilliant blue, amido black or silver stain reagents. Silver staining is more sensitive (1 ng level). However, the sensitivity of Coomassie brilliant blue G-250 is 300 ng level. It is possible to reversibly stain gels prior to blotting by a couple of methods (Rybicki, 1996).

3. Enzyme-linked Immunosorbent Assay (ELISA)

3.1 Materials and Methods

Homogenize tissue with 5 times of protein extract buffer → centrifuge 10,000 rpm 20 minutes → 0.1 ml supernatant each well → over night at 4°C → PBS with 0.5% BSA washing 3 X 3 minutes → 0.1 ml diluted primary antibody 1-2 hour at room temperature → PBS washing 3 X 3 minutes → 0.1 ml diluted secondary antibody 1-2 hour at room temperature → PBS washing 3 X 3 minutes → dye (0.2 ml pNPP) → 0.05 ml 3 N NaOH → O.D. (405 nm) measurement.

3.1.1 Extract buffer

50 mM Tris-HCl or 50 mM HEPES (pH 7.4)
150 mM NaCl
0.02% sodium azide
0.1% SDS
0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF)
0.001 mg/ml aprotinin
1% Nonidet P-40 (NP-40) or 1% Triton X-100
(The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes. PMSF is usually stored as a 10 mM or 100 mM stack solution 1.74 or 17.4 mg/ml in isopropanol at –20°C).

3.1.2 Homogenize sample under ice.

3.1.3 Centrifuge sample at 10,000 rpm for 20 minutes at 4°C.

3.1.4 Keep supernatant at -70°C until usage.

3.1.5 PBS: Phosphate-buffered saline (PBS), pH 7.4, 1000 ml (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, adjust to pH 7.4 with HCl). Add 0.5% BSA of 1% milk into PBS when washing processed. It can also use Dulbecco's PBS or try others. Instead of BSA, it can use gelatin or milk. Skim (0.5% to 1%) milk could reduce the non-specific reaction.

3.1.6 Antibody: Primary and secondary antibodies are normally 1:1000 to 1:2000 diluted by PBS and 0.1 ml each well.

3.1.7 Dye: Use alkaline phosphatase yellow (pNPP) liquid substrate as the dye for the ELISA (Derango et al. 1996). This product is supplied as a ready-to-use buffered alkaline phosphatase substrate p-nitro-phenylphosphate (pNPP). Prior to reaction with alkaline phosphatase, the substrate

should appear as a colorless to pale yellow solution. It will develop a yellow reaction product when reacted with phosphatase in microwell applications. For the end-point assays, the reaction can be stopped with 0.05 ml/well of 3 N NaOH for every 0.2 ml of substrate reaction. Following the reaction with alkaline phosphatase, a yellow reaction product forms can be read at 405 nm.

3.2 Using Polyclonal Antibodies

3.2.1 Antibody purification: Protein G column is the best for this purpose.

3.2.2 Conjugate: Making conjugate is the most important part (e.g. horseradish peroxidase).

3.2.3 96-well plate: Making the solid phase using the 96-well plate.

3.3 Buffers and other reagents

3.3.1 Plate buffer: 0.1 M Sodium carbonate buffer, pH 9.5.

3.3.2 Reaction buffer: 0.01 M Sodium phosphate buffer, pH 7.2, 0.15 M NaCl (PBS), 0.5% BSA, 0.05% thimerosal; You can also use Dulbecco's PBS or try others. Instead of BSA, you can use gelatin. Skim (0.5% to 1%) milk could reduce the non-specific reaction.

3.3.3 Washing buffer: 0.05% Tween-20, 0.01 M Sodium phosphate buffer, pH 7.2 or 0.05% Tween-20, 0.15 M NaCl.

3.3.4 Developing buffer: 0.05 M Sodium acetate buffer, pH 5.5.

3.3.5 TMB stock solution: Tetramethylbenzidine 1 mg/ml in DMSO.

3.4 Making Conjugate

3.4.1 Nakane's method.

3.4.2 Glutaraldehyde method.

3.4.3 Maleimide method.

3.5 Steps

3.5.1 2 mg Horseradish peroxidase (HRP) in 1 ml water: A.

3.5.2 21.4 mg $NaIO_4$ (never to be $NaIO_3$) in 1 ml water: B.

3.5.3 100 micro-l of B into A: Color will change to the dark green!

3.5.4 Wait for 10 min at room temperature.

3.5.5 Put into the dialysis tube (such as Molecular cut off 20,000).

3.5.6 F. Put the tube into 5 mM NaAcetate buffer, pH 4.0 in a 2 to 3 l flask.

3.5.7 Dialysis overnight: Color will change to the gold.

3.5.8 Raise the pH of the HRP solution to pH = 9.0 by the addition of 0.2 M NaCarbonate buffer, pH 9.5 (try an aliquot of 0.05 ml).

3.5.9 Mix with the antibody solution (8 mg of IgG in 1 ml), which has been pre-dialyzed to 0.01 M NaCarbonate buffer, pH 9.0 overnight.

3.5.10 Incubate the mixture for 2 hr at room temperature.

3.5.11 Put freshly prepared 0.1 ml, 0.1 M $NaHBr_4$ in water to the solution.

3.5.12 Incubate at 4 degree for 2 hr.

3.5.13 Put the mixture into a dialysis tube and dialyze against PBS overnight.

3.5.14 Now the conjugate solution is ready for use. Add thimerosal to a final concentration of 0.02% for preservation. Add glycerol to a final concentration of 10% (optional). If you stock the conjugate solution for a long period such as years, stock it at -80 degree. But, in this case, don't repeat freeze-thaw. You can stock the solution at 4 degree at least 6 months.

3.6 Preparation of ELISA Plate: This will take 2 hr to overnight. Overnight is preferable

3.6.1 Dilute antibody (IgG) by Plate buffer: 5 to 10 micro-g/ml.

3.6.2 Put the diluted antibody solution, 0.1 ml to the wells of 96-well ELISA plate.

3.6.3 Incubate for 2 hr at room temperature or overnight at 4 degree.

3.6.4 Discard the solution and wash the plate three times by washing buffer. Put 200 micro-l into wells using micro-pipette or just put the Washing buffer using some devices.

3.6.5 Discard the Washing buffer by tapping against paper towel.

3.6.6 Put 0.15 to 0.2 ml of reaction buffer. Now, the plate is ready for use. You can stock the plate at least for 6 months. Take care not to dry up the plate.

3.7 Using Monoclonal Antibodies

3.7.1 Antibody purification: Antibody purification step is the only special part comparing with materials and methods in using polyclonal antibody. For most monoclonals, except for IgM, Protein G column will be good for the practical use. If you failed by this method, confirm your procedure again before proceeding to the other methods such as DEAE column. When your monoclonal antibody is IgM, try Protamine column

combined with molecular sieving column. Others are the same as above mentioned in "Using polyclonal antibodies.

3.7.2 Try skim milk (any kind of powdered milk such as powdered milk for babies) instead of BSA: It's really cheap! Try 1% to 3%. It will decrease the background!! Thing is stability. It will form precipitate if you keep it for a few months. If you are running many plates, it is good alternative.

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