Western Blotting and ELISA Techniques

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Abstract: Western blotting 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 serum, urine and tissue/cultured cell supernatant, and they are especially widely used in protein detection. Western blotting and ELISA have widely application in scientific researches, industry and medical practice. Besides the Western blotting, Northern blotting and Southern blotting are also useful in the biochemical application. This article describes the principle techniques for Western blotting and ELISA procedure. [Researcher. 2009;1(2):67-86]. (ISSN: 1553-9865).

Keywords: assay; enzyme-linked immunosorbent assay (ELISA); method; protein; SDS-polyacrylamide gel electrophoresis (SDS-PAGE); Western blotting

Abbreviation:
2-D, two dimensions
Ab, antibody
Ag, antigen
BCIP/NBT, 5-brono-4chloro-3-indolyl phosphate/nitro blue tetrazolium
ELISA, enzyme-linked immunosorbent assay
HRP, horseradish peroxidase
NP-40, Nonidet P-40
PBS, Phosphate-buffered saline
PMSF, phenylmethylsulfonyl fluoride
PVDF, polyvinylidene fluoride
SDS, sodium dodecyl sulfate
SDS-PAGE, SDS-polyacrylamide gel electrophoresis

1. Introduction

Western blotting and enzyme-linked immunosorbent assay (ELISA) are widely used in the protein detection (Savige, 1998). The name of Western blotting, it is also called Western blot. 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, separate the proteins using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the beginning of the Western blotting. This separates the proteins by size. Second, transfer the protein from SDS-gel to a nitrocellulose membrane (electric transfer). Third, put the primary antibody on the membrane. Fourth, 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. Recently, Knudson et al used Western blotting and ELISA to measure plasma endothelin-1 and it showed that plasma endothelin-1 concentrations were not different between control and prediabetic dogs. Also, they used Western blotting method and revealed a significant decrease in endothelin-A receptor protein in left circumflex coronary arteries (Knudson, 2006). The basic principle of an ELISA is to use an enzyme to detect the binding of antigen (Ag) 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. This article describes the principle techniques for Western blotting and ELISA procedure.

Polyvinylidene fluoride (PVDF) and nitrocellulose are the two membrane types most commonly used in Western blotting applications. PVDF was first introduced as a substrate by Millipore Corporation in 1985. Nitrocellulose membrane is a high quality membrane ideal for blotting of proteins and nucleic acids. The Nitrocellulose membrane is available in two pore sizes: 0.2 μm, transfer of low molecular weight proteins (<20 kDa) and nucleic acids (<300 bp); 0.45 μm, transfer of most proteins (>20 kDa) and nucleic acids (>300 bp). In our lab, we used nitrocellulose membrane.

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 antiboy 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 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 Electronic Transfer

The polyacrylamide gel is good for separating of protein, but not suible 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

(1) 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).

(2) 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-redioctivity 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 suitble 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 Tissue Sample Preparation

2.2.1 Isolate tissue (about 1 gram).
2.2.2 Put tissue in 3 volume of extract buffer.
2.2.3 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 stock solution (1.74 or 17.4 mg/ml in isopropanol) at –20°C.
2.2.4 Homogenize sample under ice.
2.2.5 Centrifuge sample at 10,000 rpm for 10 minutes at 4°C, and collect the supernatant that contains the target protein for the measurement.
2.2.6 Keep supernatant at -70°C until usage.

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

2.3.1 Use 12% SDS gel (or 5-15% depending on the sample). 12% SDS gel preparation is shown in Table 2 and an optional 12% SDS gel

preparation reagent amount is shown in Table 3.

2.3.2 Take 50 µl 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°C).

2.3.3 Boil the sample (in loading buffer) at 100°C for 3 – 5 minutes.

2.3.4 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.

2.4 **Electronic transfer and Immunological analysis**

2.4.1 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.

2.4.2 Western blotting transfer buffer (Table 5).

2.4.3 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.

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

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

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

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

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

2.4.9 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.

2.4.10 Immediately incubate the filter with secondary antibody.

2.4.11 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.

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

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

2.4.14 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.

2.5 Alkaline phosphatase stain

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

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

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

2.5.4 Immediately stop the enzymatic reaction by add water.

2.5.5 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 (µl)

	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 (µl)

	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)	

2.6 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°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

2.7 More description of Western blotting

2.7.1 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).

2.7.2 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). 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).

2.8 A Practical Protocol for Western Blotting Handling

Reagents needed for day one:

First, prepare tissue lysis buffer and do the lysis (Table 12).

Table 12. Tissue lysis buffer for Western Blotting

Tissue Lysis Buffer for Western Blotting, all with stock solution

Chemicals	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Final con.	Notes
H_2O	6.55	19.65	32.75	65.5	98.25	131	75.60%	Room Temperature
NaCl, 5 M	0.3	0.9	1.5	3	4.5	6	0.15 M	4°C, MW=58.4
Tris-HCl, 2 M, pH 7.4	0.25	0.75	1.25	2.5	3.75	5	0.05 mM	4°C, MW=121.1
EDTA (100 mM)	0.1	0.3	0.5	1	1.5	2	1 mM	4°C, MW=372.2
Sucrose (2.5 M)	1	3	5	10	15	20	250 mM	4°C, MW=342.3
Igepal (or Triton X-100)	0.1	0.3	0.5	1	1.5	2	1%	Room Temperature
Aprotinin (1.34 mg/ml)	0.07	0.21	0.35	0.7	1.05	1.4	10 ug/ml	10 mM HEPES, -20°C
Leu-peptin (10 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	10 ug/ml	in water, -20°C
Pepstatin (1 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	1 ug/ml	in ethanol, -20°C
Trypsin inhitor (10 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	10 ug/ml	in water, -20°C
NaN_3, 2%	0.1	0.3	0.5	1	1.5	2	0.02%	in water, room temperature
NaF (50 mM)	1	3	5	10	15	20	5 mM	in ethanol, -20°C
PMSF 100 mM (17.42 mg/ml)	0.5	1.5	2.5	5	7.5	10	5 mM	add before use, -20°C
Sum	10	30	50	100	150	200		

Optional, 25 mM imidazole (MW=68.1) can be used as buffer, instead of Tris-HCl.

Tissue Lysis Buffer for Western Blotting, some reagents by powder

Chemicals	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Stock (ml)	Final con.	Notes
H_2O	7.95	23.85	39.75	79.5	119.25	159	75.60%	Room Temperature
NaCl, 5 M	0.0876 g	0.2628 g	0.438 g	0.876 g	1.314 g	1.752 g	0.15 M	MW=58.4
Tris-HCl, 2 M, pH 7.4	0.25	0.75	1.25	2.5	3.75	5	0.05 mM	4°C, MW=121.1
EDTA	0.0037 g	0.011 g	0.0186 g	0.037 g	0.0558 g	0.0744 g	1 mM	MW=372.2
Sucrose	0.8558 g	2.567 g	4.2787 g	8.557 g	12.836 g	17.115 g	250 mM	MW=342.3
Igepal (or Triton X-100)	0.1	0.3	0.5	1	1.5	2	1%	Room Temperature

Aprotinin (1.34 mg/ml)	0.07	0.21	0.35	0.7	1.05	1.4	10 ug/ml	10 mM HEPES,-20°C
Leu-peptin (10 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	10 ug/ml	in water, -20°C
Pepstatin (1 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	1 ug/ml	in ethanol, -20°C
Trypsin inhitor (10 mg/ml)	0.01	0.03	0.05	0.1	0.15	0.2	10 ug/ml	in water, -20°C
NaN_3, 2%	0.1	0.3	0.5	1	1.5	2	0.02%	in water, 4°C
NaF (50 mM)	1	3	5	10	15	20	5 mM	in ethanol, -20°C
PMSF 100 mM (17.42 mg/ml)	0.5	1.5	2.5	5	7.5	10		add before use, -20°C
Sum	10	30	50	100	150	200		

Optional, 25 mM imidazole (MW=68.1) can be used as buffer, instead of Tris-HCl.

To isolate the membrane protein, the membrane should be broken during the lysis, and the detergent Triton X-100 could be up to 5%.

Homogenized tissue could be centrifuged at 10,000 x g for 20 minutes (4°C).

2.8.1 Running buffer 1x

Prepare 1 day before, store at 4°C, good for 2 weeks.

(1) ➔ 100 ml of 10x running buffer (Bio-Rad, Cat # 161-0732, store at room temperature).
(2) ➔ 900 ml H_2O.

If 10x Tris/glycine/SDS buffer is not available you can prepare it as follows:

(1) ➔ 29 g Trizma base (Sigma T-1503 500 g).
(2) ➔ 144 g glycine (Bio-Rad 161-0717).
(3) ➔ 10 g SDS (Bio-Rad 161-0301 100 g).
(4) ➔ H_2O to 1 liter.
(5) ➔ Adjust pH = 8.3.

2.8.2 Cold transfer buffer

Prepare 1 day before, store at 4°C, good for 2 weeks.

(1) ➔ 100 ml of 10x transfer buffer (Bio-Rad, store at room temperature).
(2) ➔ 200 ml methanol.
(3) ➔ H_2O to 1 liter.

If 10x transfer buffer is not available you can prepare it as follows:

(1) ➔ 2.9 g Trizma base.
(2) ➔ 14.4 g glycine.
(3) ➔ 400 ml methanol.
(4) ➔ 1600 ml H_2O.
(5) ➔ Stir.
(6) ➔ Store in fridge until ready to use.

2.8.3 TBS-T 0.1%

Prepare 1 day before, store at 4°C, good for one week.

(1) ➔ 2 liter sterile H_2O.
(2) ➔ 4.84 g Trizma base.
(3) ➔ 16 g NaCl.
(4) ➔ Adjust pH to 7.6.
(5) ➔ Add 2 ml Tween 20 (last to add, remove pH meter prior to adding).

The preparation of TBS-T buffer is shown in Table 13.

Table 13. TBS-T 0.1% preparation

0.1% TBS-T for Western Blotting (Tris=0.02 M, pH 7.6)					
Chemicals	1000 ml	2000 ml	3000 ml	3500 ml	4000 ml
Tris base (g)	2.42	4.84	7.26	8.47	9.68
NaCl (g)	8	16	24	28	32
12 N HCl (ml)	1.29	2.57	3.85	4.49	5.13
Tween 20 (ml)	1	2	3	3.5	4
pH	7.6	7.6	7.6	7.6	7.6

Reagents needed for day two

2.8.4 5% non-fat milk (Prepare in the same day).
(1) ➔ 2.5 g of non-fat milk powder.
(2) ➔ 50 ml of 0.1% TBS-T.

2.8.5 1% non-fat milk.
(1) ➔ 0.5 g of non-fat milk powder.
(2) ➔ 50 ml of 0.1% TBS-T.

2.8.6 Get loading buffer (2X vial) out of 4°C and put it at room temperature for 1 hour.

2.8.7 Set heating block temperature to 95°C now.

2.8.8 Label 10 micro tubes, 1 to 10, for loading the wells:
(1) ➔ Tube 1: control.
(2) ➔ Tubes 2–9: samples.
(3) ➔ Tube 10: standard marker.

2.8.9 Get samples from deep freeze and place them on ice.

2.8.10 Loading buffer 2x (Use micro tubes):
(1) ➔ 380 ul Novex brand Tris-glycine SDS loading buffer (2x) small blue bottle, stored at 4°C. Let it warm up for one hour.
(2) ➔ 20 ul beta-mercaptoethanol (Bio-Rad 1610710), stored in hood (bad smell).

2.8.11 Loading buffer 1x (use micro tubes)
(1) ➔ 200 ul loading buffer 2x
(2) ➔ 200 ul running buffer 1x

2.8.12 Get loading instructions sheet (list of the specimens be worked with).

2.8.13 Get ice.

2.8.14 Label 10 micro tubes, 1 to 10, for loading the wells.
(1) ➔ Tube 1: control.
(2) ➔ Tubes 2 to 9: specimens.
(3) ➔ Tube 10: marker.
(4) ➔ Get specimens from deep freeze and place them in rack on ice.
(5) ➔ Prepare loading buffer 2x.
(6) ➔ Prepare loading buffer 1x.

When you prepare the micro tubes for loading, pipet as follows:
(1) ➔ Running buffer.
(2) ➔ Homogenate specimens.
(3) ➔ 2x loading buffer last.
(4) ➔ Mix well with vortex.

2.8.15 Heat tubes on heating block at 95°C for 1 minute (except for the marker tube).

2.8.16 Centrifuge for 1 minute.

2.8.17 Cool tubes on ice for at least 3 minute.

2.8.18 Set up your electrophoresis chamber.

2.8.19 Get one Novex gel out: remove tape at bottom of gel.

2.8.20 Rinse gel with sterile water cassette without removing the comb.

2.8.21 Now remove comb and wash gel with 1x running buffer at the sink using a pipette.

2.8.22 Place gel in electrophoresis chamber.

2.8.23 Pour 1x running buffer into middle chamber, check that it is water tight. The level must be above the loading wells of the gel and above the white bar. Get rid of any air bubbles, using a pipette.

2.**8.24 Load 20 ul of samples** into each well (clean tip with 1x running buffer between specimens).

2.8.25 Fill front and back chambers with cold 1x running buffer.

2.8.26 First electrophoresis:
(1) ➔ Set constant first.
(2) ➔ Constant: AMP.
(3) ➔ Voltage: 65 volts (or up to 90

volts).
(4) ➔ AMP: 0.02 AMP.
(5) ➔ Time: 30 minute.
(6) ➔ Press run.

After 30 min, change the setting to:
(1) ➔ Constant: AMP.
(2) ➔ Voltage: 105 volts (or up to 120 volts).
(3) ➔ AMP: 0.02 AMP.
(4) ➔ Time: 90 minutes.
(5) ➔ Press run.
(It does not take the exact 90 minutes, stop when blue line reaches the bottom).

2.8.27 During electrophoresis time, cut chromatography paper and nitro membrane into 8.5x8.5 cm.

2.8.28 Soaking
(1) ➔ Soak sponges with cold transfer buffer and remove any air trapped in them (this can be started 1 day before).
(2) ➔ Soak chromatography paper in the same container. Place the container and contents in the fridge for at lease 30 minutes at 4^0C to keep it cold.
(3) ➔ When blue line reaches middle of gel (about 60 minutes before second electrophoresis): Soak the nitro membrane in sterile H_2O for 10 minutes, then place it with the sponges and the chromatography papers in the container.

2.8.29 At the end of the first get electrophoresis:
(1) ➔ Remove gel cartridge and place it in the palm of your hand, face down, and open gently with scraper. Get rid of the back of the cartridge.
(2) ➔ Take the wet chromatography paper from the transfer buffer container and place it over the gel.
(3) ➔ Flip this upside down, the front of the cartridge is now on top.
(4) ➔ Insert putty knife to separate gel from cartridge cover. Discard front cartridge cover.
(5) ➔ Place chromatography paper which has the gel over it on a flat surface that is covered with parafilm. Cut excess perimeter of gel.
(6) ➔ Place the membrane on top of the gel. Do not allow the membrane to dry.
(7) ➔ Place the other wet chromatography paper over the membrane.
(8) ➔ Transfer this "sandwich" as is using the scraper and place it in the awaiting deep end of the gray module. It contains 2 wet sponges.

2.8.30 Note: For the electronic transfer (second electrophoresis), use the deep and thin ends of the blot module that have a gray padding.
Made the following sandwich:
(1) ➔ Sponges
(2) ➔ Sponges
(3) ➔ Sponges
(4) ➔ Chromatography paper
(5) ➔ Nitrocellulose membrane (Amersham, Hybond)
(6) ➔ Gel
(7) ➔ Chromatography paper
(8) ➔ Sponges
(9) ➔ Sponges
(10) ➔ Sponges

This sandwich sits in the blot module deep component. Sqeeze tight. Wet top of sandwich with a dropper using some cold transfer buffer.

2.8.31 Place the blot module in transfer chamber.
➔ Fill middle chamber with: cold transfer buffer from tupperware.

2.8.32 Fill front and thereby back chambers with remaining cold transfer buffer from tupperware.

2.8.33 Electronic transfer (second electrophoresis)
(1) ➔ Constant: v
(2) ➔ Voltage: 75
(3) ➔ AMP: 2
(4) ➔ Time: 90 minutes.

2.8.34 At the end of the 90 minutes, pull out the blot module, and lay aside all parts of the sandwich except for the membrane (shiny nitrocellulose membrane).

2.8.35 Grab it with large hemostat, and leave it to dry.

2.8.36 The sponges are re-usable, rinse them under faucet and save them. Discard gel and chromatography paper.

2.8.37 When the shiny membrane is dry, label the front side "f" and note which is top and bottom.

2.8.38 Blocking: Always work with the membrane face up.

(1) ➔ Add 50 ml of 5% non-fat milk into small glass container.
(2) ➔ Put in membrane, face up.
(3) ➔ Store blocked membrane 1-2 hours at room temperature or overnight at 4^0C.

2.8.39 Prepare 0.1% TBS-T (if you have not already done so).

End of day one

Day 2

2.8.40 Prepare primary antibody (1° ab) in 10 ul 0f 1% non-fat milk, normally the dilution ratio is 1/250-1/2000 (5–40 ul), just 10 minutes before use.

2.8.41 Drain blocking solution off membrane and container.

2.8.42. Pour primary antibody solution onto membrane, incubate for 1-2 hours at room temperature or overnight at 4^0C.

2.8.43 Washing:

To remove excess primary antibody (unbound). Place membrane in tupperware container.

(1) ➔ Wash with 200 ml of 0.1 % TBS-T and shake for 5 minutes.
(2) ➔ Wash with 50 ml of 0.1 % TBS-T and shake for 10 minutes.
(3) ➔ Wash with 50 ml of 0.1 % TBS-T and shake for 10 minutes.

2.8.44 Prepare secondary antibody (2° ab) in 10 ul 0f 1% non-fat milk, normally the dilution ratio is 1/500-1/5000 (2–20 ul). Place the membrane in the glass container, face up. Add 2° ab solution. Incubate for 1-2 hours at room temperature or overnight at 4^0C. This is a good time to prepare the stripping solution.

2.8.45 Washing:

To remove excess 2^0 ab (unbound). Place membrane in tupperware container.

(1) ➔ Wash with 200 ml of 0.1 % TBS-T and shake for 10 minutes.
(2) ➔ Wash with 50 ml of 0.1 % TBS-T and shake for 10 minutes.
(3) ➔ Wash with 50 ml of 0.1 % TBS-T and shake for 10 minutes.

2.8.46 Prepare chemiluminescent solution. Lights off.

Items needed for chemiluminescence:

(1) ➔ Wooden rack
(2) ➔ One 50 ml tube and two 15 ml tubes
(3) ➔ Cassette
(4) ➔ Black dish and cover
(5) ➔ Plastic wrap
(6) ➔ Scotch tape
(7) ➔ Tweezers
(8) ➔ Timer

Get the Amersham Reagent kit (RPN 2106) in the fridge.

(1) ➔ Pour 8 ml of bottle #1 in a 15 ml tube
(2) ➔ Pour 8 ml of bottle #2 in another 15 ml tube
(3) ➔ Combine and mix in 3rd tube (50 ml tube), just prior to usage.

2.8.47 Line X-ray cassette

2.8.48 Transfer the membrane into the flat black dish. Let it sit for 1 minute with the mixed reagents, no agitation.

2.8.49 Remove membrane from solution after 1 minute. Get rid of excess fluid by touching membrane. Corner onto paper towel.

2.8.50 Place membrane squarely in X-ray cassette. Cover membrane by folding up the plastic wrap. Get rid of air bubbles. Roll finger gently over plastic wrap. Tape down with scotch tape.

2.8.51 X-ray develop. If the picture you get is too light: repeat with longer exposure time. If the picture is you get is too dark: repeat with shorter exposure time.

2.8.52 Membrane stripping- prepare stripping solution

(1) ➔ 0.2 g SDS [0.2%]
(2) ➔ 0.375 g glycine [50 mM]
(3) ➔ 100 ml H_2O
(4) ➔ 200 ul HCl (12 N) [24 mM]
(5) ➔ Adjust ph to 2.6

After finishing with X-ray, you should put nitrocellulose membrane into stripping solution (however, if you need to prepare stripping solution after X-ray, place nitrocellulose membrane in 0.1% TBS-T to keep it from drying out. Once stripping solution is prepared, place membrane into stripping solution).

2.8.53 Stripping

Incubate membrane in stripping solution for 2 minutes with shaking.

2.8.54 Washing

Wash membrane: 3 times as follows:

(1) ➔ In 50 ml 0.1% TBS-T for 5 minutes.
(2) ➔ In 50 ml 0.1% TBS-T for 5 minutes.
(3) ➔ In 50 ml 0.1% TBS-T for 5 minutes.

(Optional, you could transfer membrane into fresh 0.1% TBS-T for overnight storage at 4°C).

2.8.55 Prepare primary beta-actin antibody.

(1) ➔ Sigma A5441 (-20^{0}c)
(2) ➔ Dilution: 1/10,000 - 1/1,000 (2 ul/20 ml - 10 ul/10 ml, by 1% non-fat milk).

2.8.56. Drain blocking solution off membrane.

2.8.57. Incubation:

Pour beta-actin antibody solution onto membrane. Agitate for a few minutes and incubate for one hour at room temperature or at 4^{0}C overnight.

End of day 2

Day 3

2.8.58 Washing

(1) ➔ Wash with 200 ml of 0.1 % TBS-T for 5 minutes.
(2) ➔ Wash with 50 ml of 0.1 % TBS-T for 5 minutes.
(3) ➔ Wash with 50 ml of 0.1 % TBS-T for 5 minutes.
(4) ➔ Wash with 50 ml of 0.1 % TBS-T for 5 minutes.

2.8.59 Prepare secondary antibody (2° ab) for beta-actin

(1) ➔ Amersham anti-mouse RPN 2108 (4^{0}C).
(2) ➔ Dilution is: 1/10,000 – 1/1,000.
(3) ➔ You need: 2 ul of ab + 20 ml to 10 ul of ab + 10 ml of 1% non-fat milk.
(4) ➔ Shake for 1 hour at room temperature or over night at 4^{0}C.

2.8.60 Washing (Place membrane in tupperware container):

(1) ➔ Wash with 200 ml of 0.1 % TBS-T for 5 minutes.
(2) ➔ Wash with 50 ml of 0.1 % TBS-T for 10 minutes.
(3) ➔ Wash with 50 ml of 0.1 % TBS-T for 10 minutes.
(4) ➔ Wash with 50 ml of 0.1 % TBS-T for 10 minutes.

2.8.61 Prepare chemiluminescent solution.

(1) ➔ Lights off.
(2) ➔ Get the Amersham kit, RPN 2106 in the fridge (4^{0}C).
(3) ➔ Pour ➔ 8 ml of bottle #1 in a 15 ml tube.
(4) ➔ And ➔ 8 ml of bottle #2 in another 15 ml tube.
(5) ➔ Combine and mix in 3rd tube just prior to usage.

2.8.62 Line X-ray cassette with plastic wrap

2.8.63 Transfer the membrane into the flat black dish.

Let sit for 1 minute, no agitation.

2.8.64 Remove membrane from solution after 1 minute.

Get rid of excess fluid by touching membrane corner onto paper towel.

2.8.65 Place membrane squarely in X-ray cassette. Cover membrane by folding up the plastic wrap. Get rid of air bubbles. Roll finger gently over plastic wrap. Tape down with scotch tape.

2.8.66 X-ray develop. If the picture you get is too light: repeat with longer exposure time. If the picture you get is too dark: repeat with shorter exposure time.

For beta-actin exposure time: 1 minute.

2.8.67 You can now wrap the membrane in plastic wrap and store it in the freezer, or put the membrane in 0.1 % TBS-T for 5 minutes and store at 4^0C. You have now done with the Western blot.

2.8.68 Suggested list of reagents and chemicals for western blotting test.

(1) ➔ NaCl: Sigma, S-9888.
(2) ➔ Antibodies and controls: Santa Cruz, Sigma, Pierce, etc, store at -20°C or 4°C depending on product instruction.
(3) ➔ EDTA: Sigma, E-5134.
(4) ➔ Igepal: Sigma, I-3021.
(5) ➔ PMSF (phenyl methyl sulfonyl fluoride): Sigma, P-7626, dissolved in 100% ethanol and kept at -20°C (50 ml is enough).
(6) ➔ Aprotinin: Sigma, A-1153 (concentration 1.34 mg/ml in H_2O and stored at 4°C).
(7) ➔ Leupeptin: Sigma, 151553 (concentration 10 mg/ml in H_2O and split into tubes with 20 ul each, store at -20°C.
(8) ➔ Tris-glycine SDS Novex brand 2x sample buffer: Novex LC 2676 (20 ml, blue bottle), kept at 4°C.
(9) ➔ Beta-mercaptol-ethanol: Bio-Rad, 1610710, store at room temperature.
(10) ➔ Tween 20 (polyoxyethylene): Sigma, P-1379.
(11) ➔ Trizma base: Sigma, T-1503, 500 g.
(12) ➔ Glycine: Biorad, 161-0717.
(13) ➔ Methanol, Sigma, M-1770.
(14) ➔ Chemiluminescent solution: Amersham, ECL, RPN 2106, 1-800-323-9750.
(15) ➔ Secondary antibody, Amersham, ECL, RPN 2108, anti-mouse or anti-rabbit, 4°C.
(16) ➔ Gel: Novex, EC 6035 (4-12 % Tris-glycine gel, 1.0 mm x 10 well.
(17) ➔ Nitrocellulose memrane by Hybond, Amersham, RPN 2020d (20 cm x 20 cm, 10 sheets). 0.45 micron. 1-800-323-9750.
(18) ➔ Labelon, transparency film, Labelon, XTR-660, 4.0 mil, 8.5 x 11 inches.
(19) ➔ SDS (sodium dodecylsulfate): Bio-rad, Cat# 161-0301 (100 g bottle).
(20) ➔ Non-fat milk: from normal supermarket.
(21) ➔ Multimark: Novex, LC 5725 (-20°C).

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^oC → 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.

4. Northern blotting and Southern blotting

Besides the Western blotting, Northern blotting and Southern blotting are also useful in the biochemical application. The following is a brief description of the Northern and Southern blottings.

4.1 Northern blotting

The northern blotting is a technique to detect RNA and it is used in molecular biology research to study gene expression. It takes its name from the similarity of the procedure to the Southern blotting procedure (named for biologist Edwin Southern). This technique was developed by James Alwine while working as a postdoc at Stanford University.

A notable difference in the procedure (as compared with the Southern blot) is the addition of formaldehyde in the agarose gel, which acts as a denaturant. As in the Southern blot, the hybridization probe may be made from DNA or RNA.

A variant of the procedure known as the reverse northern blot was occasionally (although, infrequently) used. In this procedure, the substrate nucleic acid (that is affixed to the membrane) is a collection of isolated DNA fragments, and the probe is RNA extracted from a tissue and radioactively labelled.

The use of DNA microarrays that have come into widespread use in the late 1990s and early 2000s is more akin to the reverse procedure, in that they involve the use of isolated DNA fragments affixed to a substrate, and hybridization with a probe made from cellular RNA. Thus the reverse procedure, though originally uncommon, enabled the one-at-a-time study of gene expression using Northern analysis to evolve into gene expression profiling, in which many (possibly all) of the genes in an organism may have their expression monitored.

(http://en.wikipedia.org/wiki/Northern_blot)

4.2 Southern blotting

A Southern blotting a technique to detect DNA. It is a method in molecular biology of enhancing the result of an agarose gel electrophoresis by marking specific DNA sequences. The method is named after its inventor, the British biologist Edwin Southern. This caused other blotting methods to be named similarly as plays on Southern's name (for example, Western blot, Northern blotting, Southwestern blotting, or Hawaiian

blotting).

4.3 Methods

The gel from the DNA electrophoresis is treated with an alkaline solution (typically containing sodium hydroxide) to cause the double-stranded DNA to denature, separating it into single strands. Denaturation is necessary so that the DNA will stick to the membrane and be hybridized by the probe. Restriction endonucleases are used to break the DNA strands into fragments.

A sheet of nitrocellulose or nylon membrane is placed on top of the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel). This causes the DNA to move from the gel onto the membrane by capillary action, where it sticks.

The membrane is then baked (in the case of nitrocellulose) or exposed to ultraviolet radiation (nylon) to permanently crosslink the DNA to the membrane.

The membrane is now treated with a hybridization probe - an isolated DNA molecule with a specific sequence that pairs with the appropriate sequence. The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, the hybridization probe may be made from RNA, rather than DNA.

After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on x-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of color on the membrane itself if a chromogenic detection is used.

Discussion

Western blotting and ELISA have widely application in scientific researches, industry and medical practice. For example, in medical, 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 indictate 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).

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