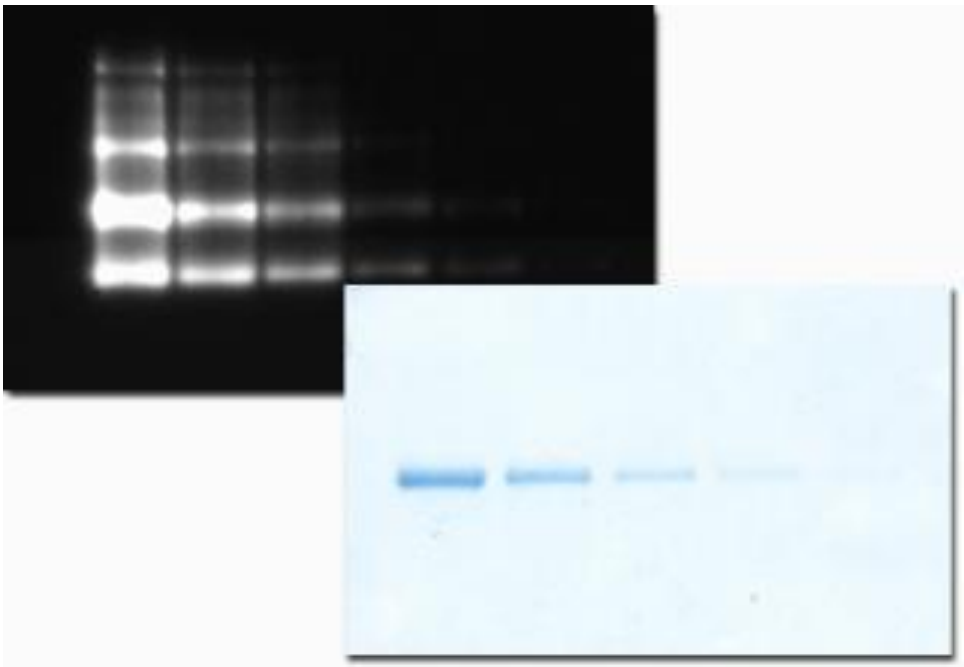
Tips for Western Blotting



ATTO BIOSCIENCE & BIOTECHNOLOGY

1. Western Blotting

Operation Flow

Electrophoresis

⇩

Western blotting

⇩

Antigen-antibody reaction, etc.

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Visualization and shooting

⇩

Analysis

Western blotting is a method by which proteins, which have been separated via gel electrophoresis, are transferred to a membrane. The reason why proteins are purposely transferred to a membrane is that the specific detection, in which only a target protein is visualized, should be carried out via antigen-antibody reaction or other reactions. If the gel were employed for detection, the separated samples (proteins) would diffuse during the detection process, a long time would be required for antibodies to enter the gel and react with the target proteins in the gel, or a large volume of reactive solution would also be required. On the other hand, when a membrane is employed, the samples (proteins) become fixed on the membrane, so that an antibody can readily react with the sample (target protein), which is exposed on the surface of membrane, requiring only a small volume of reactive solution and a short reaction time. In addition, there are fewer worries regarding damage, as often happen with gels.

This convenient western blotting method was made public in 1979. As the blotting of DNA had already been referred to as "southern" blotting, that of proteins became known as "western" blotting. In some cases, the western blotting (or western method) also includes the process of detection. Although the Towbin method, i.e. a blotting method in which proteins were electrophoretically transferred in homogeneous solution were generally used at first, a semi-dry method, made public by Kyhse-Andersen in 1984, in which the blotting solution was soaked through paper filters in order to laminate the samples, is currently frequently employed, due to its user-friendliness and economic efficiency.

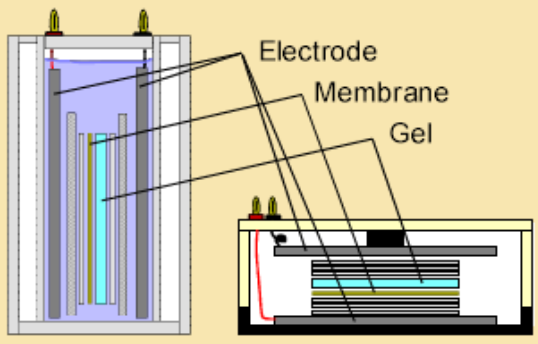
* Kyhse-Andersen, J. (1984) J. Biochem. Biophys. Methods, 10, 203-209.

2. Apparatus

The polyacrylamide gel is generally employed for protein electrophoresis, the proteins were electrophoretically transferred from the gel to the membrane, in the direction of the plane. Both a blotting apparatus and an electrical power supply are therefore required for the blotting. Two types of blotting apparatus may be employed; the first is referred to as the "tank-type," or vertical perpendicular style, while the second is referred to as the "semi-dry," or horizontal style. Our "HorizBlot" is an example of the latter type.

In the tank-type apparatus, the proteins are mildly and efficiently transferred, although there are also some disadvantages. First of all, a large volume of solution is required for use with a large gel, because the sandwich consisting of the membrane, gel, and filter papers is directly soaked in solution, with application of electricity. In addition, the blotting process requires a cooling system as the system generates substantial heat and an exclusive electrical power supply capable of generating a large current (A unit). In contrast, in the semi-dry type apparatus, only a small volume of blotting solution is used for wetting the filter papers. As excess current is not applied in the system, there is no worry of heating and thus no exclusive power supply is required.

The schematic view for the blotting apparatus



Several features such as an easy operation and a short blotting time have gained widespread acceptance, and thus a variety of chemical luminescence models are now commonly employed.

Product name	"HorizBlot"	
Model	AE-6687	AE-6688
Size of electrode	196x97mm	196x202mm

Product name	"powerStation 1000VC"
Model, power output	AE-8450, up to 500 mA, up to 1000 V

Blotting apparatus "HorizBlot"

Power supply "powerStation 1000VC"

3. Membrane and Blotting Solution

Although a nitrocellulose membrane has traditionally been used as the blotting membrane, currently, a PVDF (polyvinyliden difluoride) membrane is primarily employed. Our membrane, the "ClearBlot P membrane," is also a PVDF membrane. The observed high frequency of usage is driven by a number of desirable characteristics. In particular, the PVDF membrane: can bind a large quantity of protein (although the quantity varies by protein, the PVDF membrane exhibits a 2- to 4-fold greater capacity than nitrocellulose membranes); exhibits strong retention (i.e., once bound, proteins are difficult to remove); and, is both sturdy and easy to handle. It exhibits chemical resistance and can be applied to amino acid sequence analysis, so that the series, electrophoresis (separation) ® blotting (to membrane) ® sequence analysis (amino acid determination) is also recognized as a common procedure. Some workers cleverly employ cheaper nitrocellulose membranes, instead of a PVDF membrane for some applications. Although the front and back surfaces of our "ClearBlot P membrane" differ slightly in appearance, these sides display the same quality of material. A method exists for storing the dried membrane after blotting, but we do not recommend this method, because re-treatment for alteration of the hydrophobic properties of the PVDF membrane is required. The membrane can be stored in a refrigerator for one night if it is soaked in blocking solution, and for 1-2 days if it is wrapped in cellophane together with a small amount of blocking solution to prevent drying. If a long storage period (or other reasons) has dried the membrane, methanol treatment (e.g., soaking in methanol for 10 sec) is required, followed by soaking in any buffers (i.e., blocking solution or washing buffer), or in a buffer with detergent (Tween-20 or Triton-X) at a final concentration of 0.1% (this may sometimes require a little time).
In any case, the antigenicity of the protein on the membrane cannot be assured, so we recommend conducting the pilot experiment. After alterations to restore the membrane's hydrophilic properties, normal manipulations are carried out.

Product name	"ClearBlot P Membrane"		
Material	PVDF		
Model	AE-6665	AE-6666	AE-6667
Size	85x90mm	130x140mm	260mmx,3m
Pore size	0.2μm		

Our accessory filter paper (absorbent paper) is clean (resists contamination), fine in texture, thicker (0.9 mm thick), and exhibits a high buffer retention. If other filter paper, in particular thinner one, is employed, care is required regarding the buffer volume.

If the stacking of 2 or more sets is desired, during the blotting, use of a dialysis membrane or cellophane is required to avoid contamination (i.e., commingling of proteins in the gel, on the cathode side). Commercially available dialysis membranes or cellophanes are acceptable for this purpose.

4. Blotting Solutions

In the semi-dry type of apparatus, 3 species of solution, which respectively contain Tris, 6-aminocaproic acid, and methanol, are commonly employed as the blotting solutions. The proteins are sandwiched between the Tris and 6-aminocaproic acid ions, and transferred towards the anode plate, so that the proteins are evenly blotted. The use of these three different solutions is also regarded to generate voltage and facilitate the easy extraction of proteins from the gel. These solutions do not contain glycine, so this method is also effective for amino acid sequence analysis. Methanol is employed to enhance the adsorptivity of the proteins to the membrane. However, since methanol will also fix the proteins to the gel, using a high concentration of methanol will prevent protein extraction from the gel, resulting in reduced transfer efficiency. Although a methanol concentration of 5 to 20% is generally used, we recommend the use of 5% methanol with the PVDF membrane, due to its high absorbance. 20% methanol may be used for low molecular weight compounds, such as peptides.

The familiar Tris-glycine-methanol buffer, derived from the Towbin method using the tank-type apparatus (perpendicular style), is not most suitable for use with the semi-dry type apparatus. If use of this type of buffer is essential, we recommend using a buffer containing 100 mM Tris (25 mM in origin), 192 mM glycine, and 5% methanol (20% in origin). However, this may reduce process efficiency.

5. Operation

Example of experiment (Semi dry Blotting)

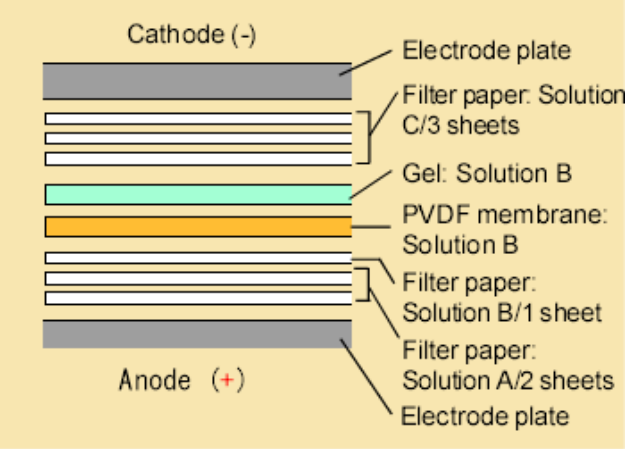
* Details are provided in the instruction manual.

Blotting solutions

Solution A: 0.3 M Tris, 5% methanol
Solution B: 25 mM Tris, 5% methanol
Solution C: 25 mM Tris, 40 mM 6-amino-caproic acid, 5% methanol

Conditions : 144 mA (2 mA/cm^2) constant current (25-35 V), 30 min

Apparatus : AE-6687 HorizBlot, AE-8450 powerStation 1000VC



Experimental Procedures

1) Prepare the blotting solution as described above. 100 mL of each solution A-C allows you to perform blotting for 1-2 sheets of a mini-gel, or 1 sheet of slab gel.
2) Cut the PVDF membrane and filter paper into same-sized pieces as the gel.
3) Moisten the membrane with methanol, and then agitate the membrane soaked in solution B, for more than 30 min.
4) Prepare filter papers soaked in each appropriate buffer, as shown in the figure above. Stack filter papers, membrane, and gel in order, on the anode plate allowing air to escape. Finally, remove any trapped air by squashing the whole surface with gloved hands, to induce adhesion of the membrane to gel (Refer to the photograph in 6-5).
5) Drop solution C onto the top of the filter, attach the cathode plate, and connect the electrical leads.
6) Set up the electric power supply, and begin to apply electricity at a constant current of 2 mA/cm^2 of gel area (in a mini gel, at a constant current of 144 mA and 50 V (At Blotting mode of powerStation)).
7) Blotting will be finished after 30-40 min. Remove the membrane and proceed to the detection step (antigen-antibody reaction).

6. Blotting Techniques

The factors described below have an effect on the blotting. If the blotting was unsuccessful, please refer to this information.

6-1. Composition of the blotting solution

In the original method of semi-dry blotting*[1], the 3 species of buffer used for the blotting are Tris, 6-aminocaproic acid and methanol. On the other hand, Tris-glycine-methanol buffer is not most suitable for use with the semi-dry type apparatus, since the buffer is derived from the Towbin method using the tank-type apparatus (perpendicular style). The proteins are sandwiched between the Tris and 6-aminocaproic acid ions, and transferred towards the anode plate, so that the proteins are evenly blotted. The use of these three different solutions is also regarded to generate voltage and facilitate the easy extraction of proteins from the gel. Although the original method calls for use of 20% methanol, we recommend the use of 5% methanol, as a high concentration of methanol prevents the extraction of proteins from gel*[2].

*1: Kyhse-Andersen, J. (1984) J. Biochem. Biophys. Methods, 10, 203-209.

6-2. Concentration of methanol (*[2])

Methanol has properties to fix proteins to gels, so a high concentration of methanol prevents the extraction of proteins from the gel. Therefore, the proteins in 5% methanol are easier to be extracted from the gel than in 10% methanol, and even easier than in 20% methanol. However, methanol is required to enhance the absorbance of the proteins to the membrane. We recommend the use of 10-20 % methanol only when low molecular weight samples (such as peptides) are to be transferred, and a nitrocellulose membrane (which has a lower adsorbance than a PVDF membrane*[3]) is used, instead of a PVDF membrane.

6-3. Blotting membrane (Transfer membrane) (*[3])

Although a nitrocellulose membrane was originally used as the blotting membrane, currently a PVDF membrane is mainly used, because PVDF membranes exhibit user-friendliness, higher adsorbance, and higher protein retention (i.e., more binding). A PVDF membrane is capable of binding a large quantity of protein with strong retention, so that the blotting efficiency is increased (the proteins are easily extracted from the gel) by reducing the concentration of methanol (so that the adsorption of proteins to the membrane is stimulated) in the blotting solution.

6-4. Power distribution

In standard semi-dry blotting, a constant current of 1-2 mA/cm^2 is applied for 30-90 min. The power voltage is set in proportion to the distance between the electrodes, so that it is difficult to achieve high reproducibility in the laminated type of semi-dry blotting and a current proportional to the area of the power distribution (gel) is applied in the method. Even if the current is increased or applied for a longer time, the efficiency of the blotting will not increase. Blotting efficiency may be reduced due to electroendosmosis, and excess heat will be generated by a voltage increase. If the efficiency is low, we recommend reviewing the blotting solutions*[6-1, 6-2, 6-6].

6-5. Pattern drift

As you know, sizes of filter papers and gel in the cathode side should be identical to avoid the pattern drift. However, it often occurs due to the insufficient contact of the gel to the blotting membrane, even if the same-sized filter papers and gel are used. In semi-dry blotting, you are required to squash well after the setup of the membrane and filter papers. All you need is proper squashing.

The gel is not broken so easily, so you should not be worried about this possibility.

6-6. In the case that the blotting still remains unsuccessful

Pzwroteins with a large molecular weight, basic proteins, glycoproteins, and lipoproteins sometimes demonstrate low efficiency in blotting. If the efficiency is not improved by applying the above methods, you should add 0.01-0.02% SDS to the blotting solution, on the cathode side. A high SDS concentration prevents the binding of the protein to the membrane. If you would like to take additional current-carrying time, you will need to stop applying power at 90 min, wet the membrane with new blotting buffer (take care to prevent the shift of membrane and gel), and apply current again. Reducing the gel concentration may be an alternative way of improving the efficacy.

7. Detection

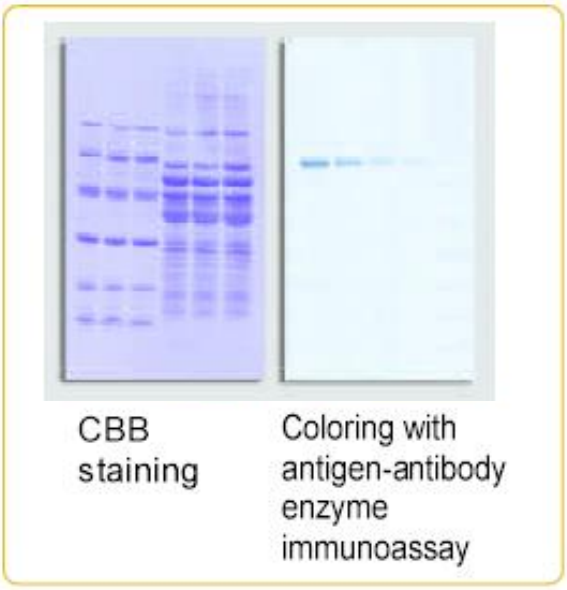
We generally detect only the target sample (protein), in other words, carry out specific detection. However, in some cases, nonspecific detection (staining) is carried out to confirm the conditions of the total sample (protein) and blotting, prior to specific detection. Staining with the CBB (Coomassie brilliant blue) dye, which is a typical method used to stain gels, is described below.

Example of the experiment (CBB staining) * Details are provided in the instruction manual.

Staining agent*1
0.001% CBB, 10% acetic acid, 30% methanol
De-coloring agent*2
10% acetic acid, 50% methanol

*1 The concentration of CBB is lower (1/10-1/100) than the ordinary reagent used for gel staining.

*2 Take care that the concentration of methanol is about 50%. (Add 45 mL of methanol in 100 mL of a mixture containing 10% acetic acid and 30% methanol, to yield a final methanol concentration of about 52%. No difficulties will result from a lower concentration of acetic acid).



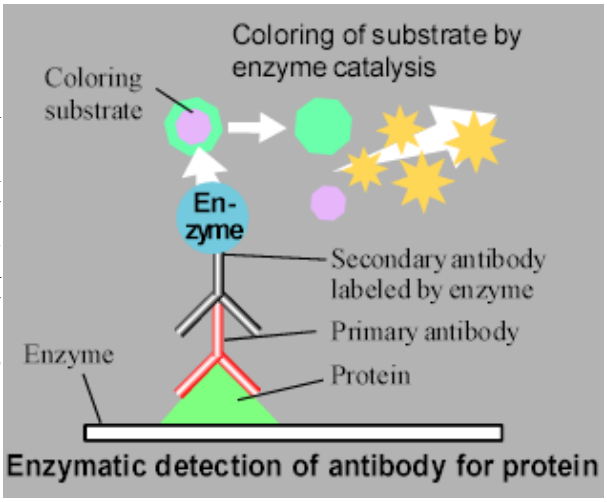
Experiment Flow

Staining,	5-10 min,	once	gently
↓			
De-coloring	2-5 min	twice	vigorously
↓			
Detection			

*Even if the background is slightly blue, the color will become white upon drying.

・Specific detection of the target protein

We generally detect only the target protein (i.e., specific detection) after the blotting. To avoid undesired behavior resulting from nonspecific binding on the membrane (undesired coloring and luminescence), we carry out blocking. Antibody for the target protein (primary antibody) is then bound after washing. The enzyme-labeled antibody, for coloring or luminescence (secondary antibody) is then continuously bound. Finally, the specific protein is detected, via coloring or luminescence by the addition of substrate.



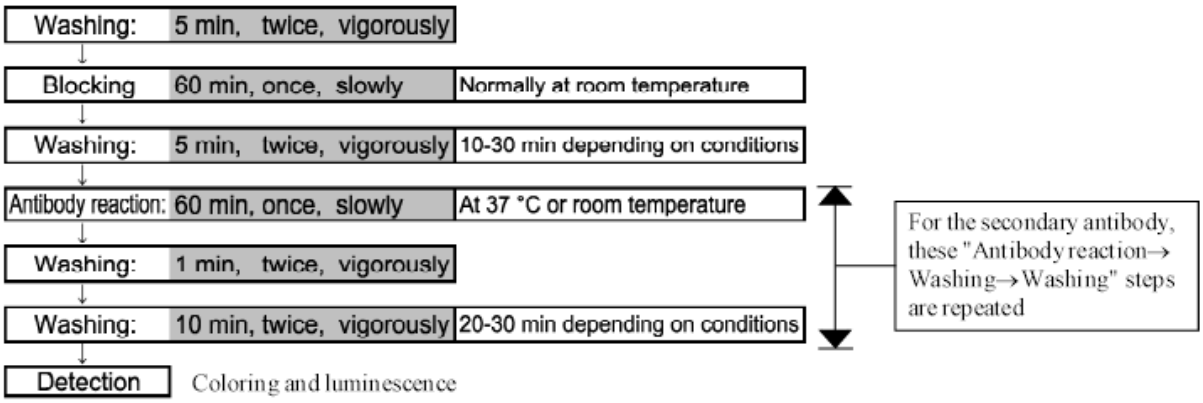
Example of experiment (Antigen-Antibody enzyme immunoassay)

* Details are provided in the instruction manual.

Blocking solution[*1]
AE-1470 EzBlok or 3% skim milk or 0.5% casein/TTBS
Washing buffer[*2]
AE-1480 EzWash + Tween-20 or TTBS (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.05% Tween-20)
(Labeled) antibody[*3]
POD (peroxidase) labeled IgG (×1/1000)/TTBS
Substrate[*4]
AE-1490 EzWestBlue Colorimetric
DAB (diaminobenzidine), H_2O_2/TE ® Colorimetric
ECL™ (Amersham, luminescent reagent)® Luminescence

*1 BSA and an exclusive reagent for blocking may also be used for the solution.
*2 To decrease the background, a detergent (Tween-20) is added to this buffer.
*3 Other enzymes, such as Alp (alkaline phosphatase) may also be used.
In detection via luminescence, the use of either 0.1% casein/TTBS or 1% skim milk/TTBS is recommended.
*4 Various substrates are available to perform both coloring and luminescence.
Note that the coloring/luminescent intensity, luminescent retention time, stability of the reagent, etc. will vary depending on the manufacturer.
* If a high background is detected, conduct the blocking at 37℃, or employ a longer wash in each step.

Experiment Flow



Luminescence Detection

Recently, the luminescence detection is frequently employed as the detection step in western blotting. When the antigen-antibody reaction is applied to western blotting, luminescent detection can be carried out via subsequent addition of a luminescent substrate, if an antibody labeled via peroxidase (POD) is employed as a secondary antibody. This luminescence is weak and invisible, so the pattern is imported with an X-ray film, or highly sensitive cooled CCD camera. Currently, the cooled CCD camera, which is easy to operate and digitize the pattern, is widely employed. This apparatus can import the luminescent pattern via fixed-time shooting in camera, following setup of the membrane with added luminescent substrate in dark room.

ATTO Cooled CCD camera system