Western Blot Protocol

CJ Xia

Abstract

Western blotting WB (immunoblotting) is a widely practiced analytical technique to detect target proteins within samples using antigen-specific antibodies. WB involves two major processes: Separation of soluble proteins into discrete bands and transfer of those proteins onto a solid matrix for subsequent analysis by immunological probes.

Under standard denaturing conditions, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate sample proteins based on polypeptide length. The separated proteins are transferred (blotted) onto a membrane matrix of nitrocellulose or PVDF where they are stained with antibody probes specific to target protein antigens. Typically, blots are incubated with antibodies against the antigen of interest followed by a secondary antibody and detection.

Analysis of protein migration and the intensity of chromogenic, chemiluminescent, or fluorescent signals offer protein expression details from cells or tissue homogenates. When coupled with high-resolution gel electrophoresis, antibodies of strong avidity and specificity to target antigens, and robust signal reporting, Western blotting can detect picogram quantities of protein.

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Protocol

Sample Preparation - Cell Culture Protein Extraction

Step 1.
Culture cells in the cell culture dish until 80% confluency.

Sample Preparation - Cell Culture Protein Extraction

Step 2.

Place the dish on ice and rinse the cells in ice-cold PBS buffer (10 mM Na_2HPO_4 and 1.8 mM $Na_2H_2PO_4$ with 0.2% Tween 20; pH 7.4).

Sample Preparation - Cell Culture Protein Extraction

Step 3.
Aspirate the PBS and add ice-cold lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl and protease inhibitors).

Sample Preparation - Cell Culture Protein Extraction

Step 4.

Scrape adherent cells off the dish using a cold plastic cell scraper or digest the cells with 0.05% trypsin cells with PBS wash. *Be as gentle as possible with the cells to avoid inducing cell stress pathways.*

Sample Preparation - Cell Culture Protein Extraction

Step 5.

Centrifuge the cells at 3,000 rpm at 4°C for 2-3 min.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Cell Culture Protein Extraction

Step 6.

Remove the supernatant and wash it 2 times with ice-cold PBS buffer (AR0030, Boster Bio).

REAGENTS

Phosphate Buffered Saline (PBS) Powder AR0030 by Boster Bio

Sample Preparation - Cell Culture Protein Extraction

Step 7.

Gently transfer the cell precipitate into an ice-cold tube.

Sample Preparation - Cell Culture Protein Extraction

Step 8.

Add Mammalian Cell Protein Extraction Reagent (AR0103, Boster Bio) into the tube (v/v: 6/1: extraction reagent/cell precipitate) and resuspend vigorously.

REAGENTS

Mammal Cell Protein Extraction Reagent AR0103 by Boster Bio

Sample Preparation - Cell Culture Protein Extraction

Step 9.

If the above solution is murky, sonicate it for 10-15 seconds to break up the proteins.

Sample Preparation - Cell Culture Protein Extraction

Step 10.

Lyse the cells in RIPA lysis buffer (AR0105, Boster Bio) on ice for 4-5 hours.

REAGENTS

RIPA Lysis Buffer AR0105 by Boster Bio

Sample Preparation - Cell Culture Protein Extraction

Step 11.

Sonicate and lyse again if the cell solution remains murky.

Sample Preparation - Cell Culture Protein Extraction

Step 12.

Centrifuge at 10,000 rpm at 4°C for 10 minutes. The centrifugation force and time can be varied depending on the cell type.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Cell Culture Protein Extraction

Step 13.

Discard the lipid (at top) and the cell debris (at bottom) by aliquotting the solution in the middle to a fresh tube and keeping the tube at -20°C.

TEMPERATURE

-20 °C Additional info:

Sample Preparation - Tissue Protein Extraction

Step 14.

Place surgically resected tissues in pre-cooled (4°C) normal saline. Make sure to wash off any blood from the tissues.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Tissue Protein Extraction

Step 15.

Cut the tissue into small pieces (0.1 g to 1 g each).

Sample Preparation - Tissue Protein Extraction

Step 16.

Add Broad Spectrum Protease Inhibitor Cocktail (AR1182, Boster Bio).

REAGENTS

Broad Spectrum Protease Inhibitor Cocktail (100x) AR1182 by Boster Bio

Sample Preparation - Tissue Protein Extraction

Step 17.

Add 10 mL Mammal Tissue Protein Extraction Reagent (AR0101, Boster Bio) per 1 g of tissue.

REAGENTS

Mammalian Tissue Protein Extraction Reagent AR0101 by Boster Bio

Sample Preparation - Tissue Protein Extraction

Step 18.

Mince the tissue and place the minced tissue in tissue homogenizer.

Sample Preparation - Tissue Protein Extraction

Step 19.

Add ice-cold lysis buffer (For a 5 mg piece of tissue, add 300 µL of buffer. Buffer volume should be determined in relation to the amount of tissue present).

Sample Preparation - Tissue Protein Extraction

Step 20.

Lyse the tissue homogenate on ice for 4-5 hours or at 4°C (high speed) for 5 minutes.

Sample Preparation - Tissue Protein Extraction

Step 21.

If necessary, sonicate until no tissue chunks remain.

Sample Preparation - Tissue Protein Extraction

Step 22.

Centrifuge at 10,000 rpm at 4°C for 10 minutes. The centrifugation force and time can be varied depending on the sample type.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Tissue Protein Extraction

Step 23.

Discard the lipid (at top) and the cell debris (at bottom) by aliquotting the solution in the middle to a fresh tube and keeping the tube at -20°C.

TEMPERATURE

-20 °C Additional info:

Sample Preparation - Protein Quantitation Assay

Step 24.

The test tube protocol for our BCA (Bicinchoninic Acid) Protein Assay Kit (AR0146, Boster Bio) is described here. Refer to our Micro BCA Protein Assay Kit datasheet (AR1110, Boster Bio; datasheet) for the microplate protocol where appropriate.

REAGENTS

BCA Protein Assay Kit AR0146 by Boster Bio

Micro BCA Protein Assay Kit AR1110 by Boster Bio

LINK:

https://www.bosterbio.com/media/pdf/AR1110_DS.pdf

Sample Preparation - Protein Quantitation Assay - Reagent Preparation

Step 25.

Reconstitute the albumin standard ampules (BSA) with 0.9% NaCl or PBS to create a working solution of 2000 µg/mL (Tube A).

Sample Preparation - Protein Quantitation Assay - Reagent Preparation

Step 26.

Mix thoroughly 50 mL BCA Reagent A with 1 mL BCA Reagent B (i.e. Diluent).

Sample Preparation - Protein Quantitation Assay - Reagent Preparation

Step 27.

Prepare the diluted BSA standards by mixing the diluent and BSA as follows:

Tube	Diluent Volume (µL)	BSA Volume (µL)	BSA Concentration (µg/mL)
A	0	600 (From Tube A)	2000
B	100	300 (From Tube A)	1500
C	300	300 (From Tube A)	1000
D	200	200 (From Tube B)	750
E	300	300 (From Tube C)	500
F	300	300 (From Tube E)	250
G	300	300 (From Tube F)	125
H	400	100 (From Tube G)	25
I (Blank)	300	0	0

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 28.

For the test-tube protocol, the ratio of sample to working range is 1:20.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 29.

Pipette 0.1 mL of each standard and unknown sample replicate into an appropriately labeled test tube.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 30.

Add 2.0 mL of the working range (WR) to each tube and mix well.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 31.

Cover and incubate tubes with one of the protocols:

- Standard Protocol: 37°C for 30 minutes (WR: 25 to 2,000 µg/mL).
- RT Protocol: Room temperature for 2 hours (WR: 25 to 2,000 µg/mL).
- Enhanced Protocol: 60°C for 30 minutes (WR: 5 to 250 µg/mL).

NOTES

CJ Xia 22 Dec 2017

Increasing the incubation time and temperature can increase the net 562 nm absorbance for each test and decrease both the minimum detection level of the reagent and the working range of the protocol. Use a water bath to heat the tubes for either the Standard (37°C incubation) or the Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 32.

Cool all tubes to room temperature.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 33.

With the spectrophotometer set to 562 nm, “zero” the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

NOTES

CJ Xia 22 Dec 2017

Because the BCA Assay does not reach a true end point, color development will continue even after cooling to room temperature. However, because the rate of color development is low at room temperature, no significant error will be introduced if the 562 nm absorbance measurements of all tubes are made within 10 minutes of each other.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 34.

Subtract the average 562 nm absorbance measurement of the blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.

Sample Preparation - Protein Quantitation Assay - Quantitation Assay

Step 35.

Prepare a standard curve by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Electrophoresis - Gel Preparation

Step 36.

The first step of gel preparation is to determine the gel percentage based on the molecular weight of your protein sample:

Protein Size (kDa)	**>200**	**15-200**	**10-70**	**12-45**	**4-40**
Gel Percentage	6%	8%	10%	12%	20%

If you are not sure of the size of your protein or are looking at proteins of a variety of molecular weights, then a gradient gel may provide the best resolution.

REAGENTS

SDS-PAGE Gel Preparation Kit AR0138 by Boster Bio

NOTES

CJ Xia 22 Dec 2017

- We recommend using the SDS-PAGE Gel Preparation Kit is available from us (AR0138, Boster Bio). It contains most of the reagents for the gel preparation and can be used to make both SDS-PAGE gel and non-native PAGE gel, respectively.

CJ Xia 22 Dec 2017

- Many protocols are available for gel preparation. Please refer to the manufacturer's guidelines for use of specific products.

CJ Xia 22 Dec 2017

- Pre-cast gels may also be used instead of making your own gel.

Electrophoresis - Gel Preparation - Resolving Gel Preparation

Step 37.

Determine volume needed and gently mix the ingredients for the chosen percentage of the resolving gel.

Electrophoresis - Gel Preparation - Resolving Gel Preparation

Step 38.

Pour the solution into your gel casting form.

Electrophoresis - Gel Preparation - Resolving Gel Preparation

Step 39.

Layer the top of the gel with distilled water.

Electrophoresis - Gel Preparation - Resolving Gel Preparation

Step 40.

Wait approximately 30 minutes for the gel to polymerize completely.

Electrophoresis - Gel Preparation - Resolving Gel Preparation

Step 41.

Remove the water from the polymerized resolving gel (absorb excess water with paper towel).

Electrophoresis - Gel Preparation - Stacking Gel Preparation

Step 42.

Determine the volume needed. Gently mix the ingredients and pour the stacking gel on top of the running gel.

Electrophoresis - Gel Preparation - Stacking Gel Preparation

Step 43.

Insert sample comb (avoid bubbles).

Electrophoresis - Gel Preparation - Stacking Gel Preparation

Step 44.

Allow 30 to 60 minutes for complete gel polymerization.

Electrophoresis - Pre-electrophoresis Sample Preparation

Step 45.

Mix the extracted protein sample with 4X Dual Color Protein Loading Buffer (AR1142, Boster Bio) at 3:1 ratio (i.e. add 300µg sample to 100µL Loading Buffer).

REAGENTS

Dual Color Protein Loading Buffer 4X AR1142 by Boster Bio

LINK:

https://www.bosterbio.com/media/pdf/AR1142_DS.pdf

NOTES

CJ Xia 22 Dec 2017

Dual Color Protein Loading Buffer is designed to prevent protein degradation during sample heating prior to electrophoresis and is able to work against pH changes during the SDS-PAGE run. Many proteins are sensitive to pH changes that result from temperature fluctuations of Tris buffers during electrophoresis.

It contains 2 tracking dyes: blue (Bromophenol Blue) for tracking the progress of electrophoresis and pink (Pyronin Y) for monitoring protein transfer to the membrane. Refer to the datasheet on our website for more information.

Electrophoresis - Pre-electrophoresis Sample Preparation

Step 46.

You may also use one of the following reagents/methods instead of the Dual Color Protein Loading Buffer:

- 2X SDS-PAGE Protein Loading Buffer (AR0131, Boster Bio) at 1:1 ratio (i.e. add 100 µg sample to 100 µL Loading Buffer)
- 5X SDS-PAGE Protein Loading Buffer (AR1112, Boster Bio) at 4:1 ratio (i.e. add 400 µg sample to 100 µL Loading Buffer)
- 2X SDS-PAGE Tricine Protein Loading Buffer (AR1143, Boster Bio) at 1:1 ratio (i.e. add 100 µg sample to 100 µL Loading Buffer) if detecting protein with MW < 10 kDa
- Laemmli 2X Buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl; pH 6.8) at 1:1 ratio (i.e. add 100 µg sample to 100 µL Loading Buffer)

REAGENTS

SDS-PAGE Loading Buffer 2X AR0131 by Boster Bio

SDS-PAGE Protein Loading Buffer 5X (Reducing) AR1112 by Boster Bio

SDS-PAGE Tricine Loading Buffer AR1143 by Boster Bio

Electrophoresis - Pre-electrophoresis Sample Preparation

Step 47.

Denature the sample/loading buffer mixture in a 100°C water bath for 5 minutes (or follow the manufacturer instructions). Alternatively, the mixture can be stored in aliquots at -20°C for several months or at 4°C for 1-2 weeks before use.

Electrophoresis - Loading Samples and Running Electrophoresis

Step 48.

Place the gel in the electrophoresis apparatus.

Electrophoresis - Loading Samples and Running Electrophoresis

Step 49.

Fill both buffer chambers with SDS-PAGE Electrophoresis Buffer (25 mMTris base, 190 mM glycine and 0.1% SDS; pH 8.3); We recommend using our buffer (AR0139, Boster Bio).

REAGENTS

SDS-PAGE Electrophoresis Buffer AR0139 by Boster Bio

Electrophoresis - Loading Samples and Running Electrophoresis

Step 50.

Carefully remove the well-creating comb from the gel and rinse wells with the electrophoresis buffer.

Electrophoresis - Loading Samples and Running Electrophoresis

Step 51.

Pipette your samples into the wells quickly to prevent possible sample diffusion inside the well (For a well with maximum 30 µL, load 20 to 25 µL of 1 µg/µL sample per well).

Electrophoresis - Loading Samples and Running Electrophoresis

Step 52.

Pipette 10 µL of appropriate controls and/or molecular weight standards in separate well(s).

Electrophoresis - Loading Samples and Running Electrophoresis

Step 53.

Connect the anode and cathode of the electrophoresis appropriately.

Electrophoresis - Loading Samples and Running Electrophoresis

Step 54.

Turn on the power to run electrophoresis at 100V/130V* until the bromophenol blue dye reaches the gel bottom (This can take 1.5 to 3 hours). You should observe fine bubbles from the gel apparatus bottom as this observation indicates sufficient electric current is generated.

NOTES

CJ Xia 03 Jan 2018

- In a discontinuous system, the electrophoresis voltage for stacking gel is lower than that for resolving gel to ensure that proteins are concentrated on the same level before running into the resolving gel.

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* The applied voltage should be adjusted according to the gel thickness, the power supply used, and the resolution desired.

Electrophoresis - Loading Samples and Running Electrophoresis

Step 55.

Turn off the power when the protein samples have finished migrating in the gel.

Protein Transfer (To Membrane) - Gel Staining (Optional)

Step 56.

After electrophoresis, we recommend using one of our gel staining solutions to determine if the electrophoretic separation works. Refer to the datasheet(s) on our website for more information.

- Coomassie Blue Staining & Destaining Solution (AR0140, Boster Bio)
- Coomassie Blue Fast Staining Solution (AR0170, Boster Bio)
- Protein Silver Staining Reagents (AR0171, Boster Bio)

REAGENTS

Coomassie Blue Staining & Destaining Solution AR0140 by Boster Bio

Coomassie Blue Fast Staining Solution AR0170 by Boster Bio

Protein Silver Stain Kit AR0171 by Boster Bio

NOTES

CJ Xia 03 Jan 2018

Stained gel cannot be used in the subsequent protein transfer procedure.

Protein Transfer (To Membrane) - Wet Transfer - Blotting Membrane Preparation

Step 57.

Cut the blotting membrane (NC or PVDF) according to the size of your gel.

NOTES

CJ Xia 03 Jan 2018

Tips: Cut a good supply of membranes in advance! Store in a cool, dry place.

Protein Transfer (To Membrane) - Wet Transfer - Blotting Membrane Preparation

Step 58.

Carefully mark the membrane orientation by cutting a corner or marking it with a pencil.

Protein Transfer (To Membrane) - Wet Transfer - Blotting Membrane Preparation

Step 59.

Soak the membrane in methanol for 1 minute.

Protein Transfer (To Membrane) - Wet Transfer - Blotting Membrane Preparation

Step 60.

Immerse the membrane in 5 minutes with 1X transfer buffer (25 mMTris base, 190 mM glycine and 20% methanol; pH 8.3) (AR1149, Boster Bio) and rock the membrane gently until it sinks and the water no longer beads up on the surface.

REAGENTS

Tris-glycine-SDS Buffer AR1149 by Boster Bio

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette

Step 61.

Based on a sandwich model, install the electric transfer cassette in the following order:
Foam Pad → Filter Paper → Gel → Membrane→ Filter Paper→ Foam Pad

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette

Step 62.

We recommend using one of the transfer pads from Boster:

- Fast and Efficient Transfer Pad, 12.5 cm X 12.5 cm (AR0172, Boster Bio)
- Fast and Efficient Transfer Pad, 9 cm X 7.5 cm (AR0173, Boster Bio)

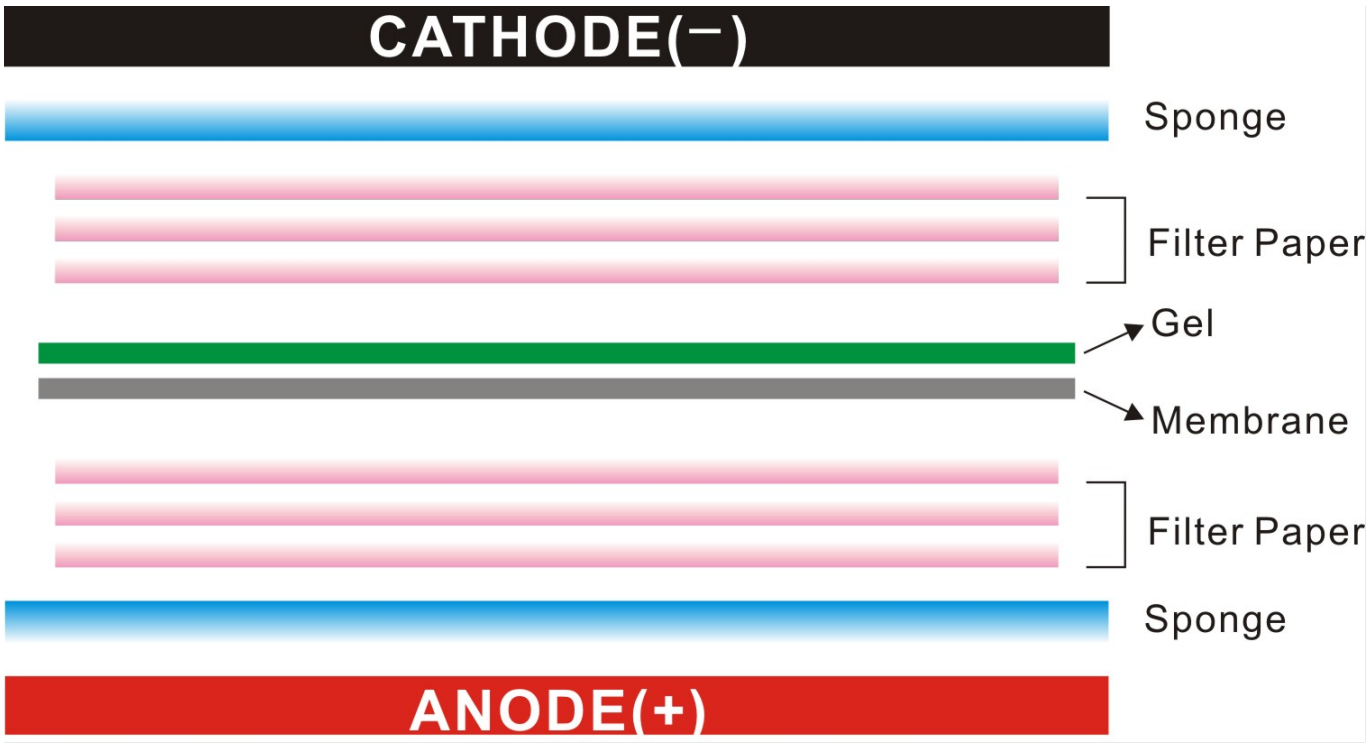
REAGENTS

Fast & Efficient Transfer Pad (12.5 cm x 12.5 cm) AR0172 by Boster Bio

Fast & Efficient Transfer Pad (9 cm x 7.5 cm) AR0173 by Boster Bio

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette

Step 63.



Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette

Step 64.

Soak two filter papers in a separate container with the same transfer buffer.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette

Step 65.

Crack open the transfer cassette with a spatula and make sure to loosen the cassette hold all the way around before carefully pulling apart the two halves.

NOTES
CJ Xia 04 Jan 2018

Before doing anything with the gel, such as cutting it, pay careful attention to the location of lane #1.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 66.

Cut the gel according to the size of the membrane with a razor blade and then cut the corner on the side of the gel with lane #1.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 67.

Immerse the gel in 1X transfer buffer for 15-30 minutes.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 68.

Place the gray or black plate of the transfer cassette on a clean surface.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 69.

Place one pre-wetted foam pad on the gray side of the cassette.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 70.

Place a moistened sheet of filter paper on the foam pad.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 71.

Carefully peel the gel off of the remaining half of the gel cassette and place it on the filter paper.

NOTES
CJ Xia 04 Jan 2018

Moisten the gel with transfer buffer and use a serologically clean pipette or a Falcon tube, as if it were a rolling pin, to roll air bubbles out of the membrane.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 72.

Place the membrane onto the gel with the corners matched up.

NOTES
CJ Xia 04 Jan 2018

Once the membrane contacts the gel, it should not be moved or “ghost bands” can result.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 73.

Complete the sandwich by placing a piece of filter paper onto the membrane.

Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 74.

Add the second foam pad on top of the filter paper.

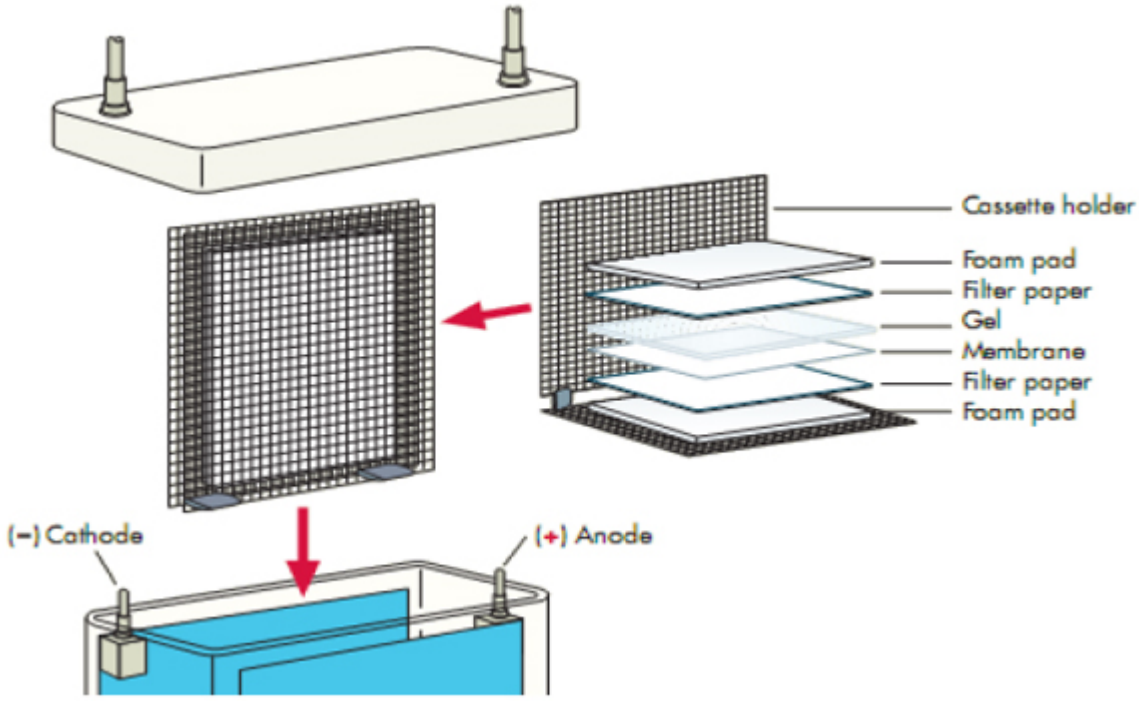
Protein Transfer (To Membrane) - Wet Transfer - Transfer Cassette
Step 75.

Lock the transfer cassette firmly with the white latch.

NOTES
CJ Xia 04 Jan 2018

Be careful not to move the gel and the filter paper sandwich. Make sure that the foam pads, filter papers, and membrane are thoroughly immersed in the transfer buffer.

Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run
Step 76.



Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run

Step 77.
Fill transfer tank with an adequate amount of 1X transfer buffer.

Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run

Step 78.
Firmly insert transfer cassette into the slot of the transfer apparatus.

Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run

Step 79.

Place the lid on top of the transfer tank and make sure the electrodes are lined correctly.

NOTES
CJ Xia 04 Jan 2018

The gel should be closer to the cathode and the membrane should be closer to the anode. Negatively charged proteins will migrate towards the anode.

Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run

Step 80.

Set the power source to constant voltage and operate at 25 V for 30 minutes.

NOTES
CJ Xia 04 Jan 2018

- The transfer can be completed overnight at a lower voltage, for example, 10 V.

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Transfer time and voltage should be optimized according to the gel concentration. Higher gel concentration requires additional time.

Protein Transfer (To Membrane) - Wet Transfer - Protein Transfer Run

Step 81.

Check the protein transfer efficiency by membrane staining:

Place the membrane in Ponceau S staining (0.2% w/v Ponceau S; 5% glacial acetic acid) or our Imprinted Membrane Fast Reversible Protein Staining Reagent (AR0142, Boster Bio) for 5-10 minutes. A visible red band will appear. The membrane may be de-stained completely by repeatedly washing in wash buffer.

REAGENTS
Imprinted Membrane Fast Reversible Protein Staining Reagent AR0142 by Boster Bio

Membrane Blocking

Step 82.

Rinse the blotting membrane 3X using TBS Wash Buffer (20 mMTris, pH 7.5; 150 mMNaCl; 0.05%

Tween 20) (AR0144, Boster Bio) at room temperature for 10 minutes each time.

REAGENTS
TBS Wash Buffer AR0144 by Boster Bio

Membrane Blocking

Step 83.

After rinsing, immerse the blotting membrane in TBS Blocking Buffer (5% non-fat dry milk in buffer of 20 mMTris, pH 7.5; 150 mMNaCl) (AR0143, Boster Bio) and incubate for 1.5 - 2 hours at room temperature (or overnight 4°C) with shaking.

REAGENTS
TBS Blocking Buffer AR0143 by Boster Bio

NOTES
CJ Xia 04 Jan 2018

Alternatively, buffer containing non-fat dried milk, gelatin, or BSA can be used. For use with biotin systems or detection of phosphoproteins, non-fat dried milk is not recommended.

Antibody Incubation

Step 84.

After blocking, the membrane is incubated with a primary antibody (that binds to the target protein) followed by an HRP-conjugated or AP-conjugated secondary antibody.

LINK:
https://www.bosterbio.com/catalogsearch/result/?q=HRP+secondary+antibody

Antibody Incubation

Step 85.

Dilute the primary antibody with the TBS Wash Buffer (AR0144, Boster Bio). Follow the antibody protocol from the manufacturer for optimal dilution.

REAGENTS
TBS Wash Buffer AR0144 by Boster Bio

Antibody Incubation

Step 86.
Incubate the primary antibody and the membrane at 4°C overnight or for 1-2 hours at room temperature. For the best results, incubation time and antibody concentration may need to be optimized.

Antibody Incubation

Step 87.

Wash the membrane 3X with the TBS Wash Buffer (AR0144, Boster Bio) for 10 minutes each to remove unbound antibody.

REAGENTS

TBS Wash Buffer AR0144 by Boster Bio

Antibody Incubation

Step 88.

Dilute the secondary antibody with the TBS Blocking Buffer (AR0143, Boster Bio). Follow the antibody protocol from the manufacturer for optimal dilution.

REAGENTS

TBS Blocking Buffer AR0143 by Boster Bio

Antibody Incubation

Step 89.

Incubate the secondary antibody and the membrane at 4°C overnight or 1-2 hours at room temperature on a shaker.

Antibody Incubation

Step 90.

Wash the membrane 3X with the TBS Wash Buffer for 10 minutes each to remove unbound antibody.

Signal Detection

Step 91.

In this section, we provide the protocols for the **Enhanced Chemiluminescence Detection (ECL)** and **Colorimetric Detection (DAB)** methods. Use the method that fits your preferences and criteria.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - ECL Substrate Preparation

Step 92.

Choose the correct ECL kit† according to the species that the primary antibody is raised.

Origin of Primary Antibody Species	Catalog No. of ECL Kit*
Mouse IgG	EK1001
Rabbit IgG	EK1002
Goat IgG	EK1003
Rat IgG	EK1004
Mouse IgM	EK1005

* Each kit has sufficient reagents for 800 cm^2 of membrane.

The ECL kit provides:

1) Chromogenic reagents A and B (20X concentrated; 5 mL)

2) Blocking buffer

3) HRP-conjugated secondary antibody

† Instead of using the ECL kit, one may use one of the following standalone chromogenic reagents A and B from Boster:

	Reagent A		Reagent B		
Product	Concentration	Volume	Concentration	Volume	Catalog No.
Hypersensitive ECL Substrate	1X**	100 mL	1X**	100 mL	AR1170
Hypersensitive WB Substrate	20X	5 mL	20X	5 mL	CR0001-5
Hypersensitive WB Substrate	20X	10 mL	20X	10 mL	CR0001-10
Hypersensitive WB Substrate	20X	25 mL	20X	25 mL	CR0001-25

** Ready-to-use

REAGENTS

Enhanced Chemiluminescent (ECL) Detection Kit (Mouse IgG) EK1001 by Boster Bio

Enhanced Chemiluminescent (ECL) Detection Kit (Rabbit IgG) EK1002 by Boster Bio

Enhanced Chemiluminescent (ECL) Detection Kit (Goat IgG) EK1003 by Boster Bio

Enhanced Chemiluminescent (ECL) Detection Kit (Rat IgG) EK1004 by Boster Bio

Enhanced Chemiluminescent (ECL) Detection Kit (Mouse IgM) EK1005 by Boster Bio

Hypersensitive ECL Chemiluminiscence Substrate AR1170 by Boster Bio

Hypersensitive WB Chemiluminescent Substrate CR0001-5 by Boster Bio

Hypersensitive WB Chemiluminescent Substrate CR0001-10 by Boster Bio

Hypersensitive WB Chemiluminescent Substrate CR0001-25 by Boster Bio

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - ECL Substrate Preparation

Step 93.

Prepare the ECL substrate solution by mixing the following and use the solution within 2 hours of preparation:
- 50 µL of 20X Chromogenic Reagent A (Luminol & Luminious Enhancer)
- 50 µL of 20X Chromogenic Reagent B (Peroxidase & Stabilizer)
- 1 mL of distilled water

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Membrane Treatment

Step 94.

Thoroughly cover the membrane with the substrate solution (use 1 mL of solution for 10 cm^2 of the membrane).

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Membrane Treatment

Step 95.

Incubate the membrane at room temperature until bands appear (usually 1-5 minutes; incubation time can be estimated in dark room).

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Membrane Treatment

Step 96.

Gently blot the edge of the membrane on a piece of paper to remove excess substrate solution.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Membrane Treatment

Step 97.

Put a clear preservative film or transparent glass paper over the membrane and remove any air bubbles observed.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 98.

Develop and fix the film in a dark room immediately using our recommended WB Developing and Fixing Kit (AR0132, Boster Bio). Alternatively, fluorescence CCD scan, digital imager or luminometer can be used.

REAGENTS

WB Developing Fixing Kit AR0132 by Boster Bio

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 99.

Put the X-ray film over the membrane.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 100.

Develop the film by immersing it in developing solution for 10 seconds to 10 minutes. Determine the exposure time required by observing under red light and stop developing once the film achieves the experimental purpose. Multiple exposures may be necessary for the optimal signal to noise ratio.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 101.

Wash the film with clean water (to remove the developing solution completely) and stop washing when bands appear.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 102.

Immerse the film in fixing solution for 3-5 minutes.

Signal Detection - Enhanced Chemiluminescence Detection (ECL) - Film Development and Fixing

Step 103.

Wash the film with clean water to remove the fixing solution.

REAGENTS

WB Stripping Buffer AR0153 by Boster Bio

NOTES

CJ Xia 04 Jan 2018

- WB Stripping Buffer (AR0153, Boster Bio) is recommended to remove primary and secondary antibodies on the membrane if proteins on the membrane need to be reused.

CJ Xia 04 Jan 2018

- Use the control protein levels to normalize the target protein levels.

Signal Detection - Colorimetric Detection - DAB Substrate Preparation (For HRP-conjugated secondary antibodies)

Step 104.

Choose the correct DAB kit according to the species that the primary antibody is raised and the desirable color:

Origin of Primary Antibody Species	Color	Catalog No. of DAB Kit
Mouse IgG	Yellow	SA2020
Goat IgG	Yellow	SA2021
Rabbit IgG	Yellow	SA2022
Rat IgG	Yellow	SA2023
Mouse IgG	Blue	SA2024
Rabbit IgG	Blue	SA2025

REAGENTS

Western Blotting Mouse IgG DAB Chromogenic Reagent Kit (Yellow) SA2020 by Boster Bio

Western Blotting Goat IgG DAB Chromogenic Reagent Kit (Yellow) SA2021 by Boster Bio

Western Blotting Rabbit IgG DAB Chromogenic Reagent Kit (Yellow) SA2022 by Boster Bio

Western Blotting Rat IgG DAB Chromogenic Reagent Kit (Yellow) SA2023 by Boster Bio

Western Blotting Mouse IgG DAB Chromogenic Reagent Kit (Blue) SA2024 by Boster Bio

Western Blotting Rabbit IgG DAB Chromogenic Reagent Kit (Blue) SA2025 by Boster Bio

Signal Detection - Colorimetric Detection - DAB Substrate Preparation (For HRP-conjugated secondary antibodies)

Step 105.

Prepare the DAB substrate solution by mixing the following:
- 50 µL of 40X Chromogenic Reagent A (DAB)
- 50 µL of 40X Chromogenic Reagent B (H_2O_2)
- 50 µL of 40X Chromogenic Reagent C (TBS Wash Buffer)
- 2 mL of distilled water

Signal Detection - Colorimetric Detection - BCIP/NBT Substrate Preparation (For AP-conjugated secondary antibodies)

Step 106.

Prepare the BCIP/NBT substrate solution by mixing the following:
- 50 µL of 20X Chromogenic Reagent A (BCIP/NBT)
- 50 µL of 20X Chromogenic Reagent B (Tris concentrated buffer, pH 9.4)
- 1 mL of distilled water

Signal Detection - Colorimetric Detection - Membrane Treatment

Step 107.

Thoroughly cover the membrane with the substrate solution (use 1 mL of solution for 10 cm^2 of the membrane).

Signal Detection - Colorimetric Detection - Membrane Treatment

Step 108.

Incubate the membrane at room temperature until bands appear (usually 10-30 minutes). Incubation for BCIP/NBT should be done in the dark.

Signal Detection - Colorimetric Detection - Membrane Treatment

Step 109.

Wash the membrane in distilled water to stop the reaction.

Signal Detection - Colorimetric Detection - Membrane Treatment

Step 110.

Observe the bands and take pictures.