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U Michigan - Western Blot V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.56hg9b6

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ABSTRACT

Summary:

Immunoblotting (also called Western Blotting) is a technique to separate and identify individual proteins in a protein mixture (e.g. a cell lysate). In Immunoblotting (Western blotting) the protein mixture is applied to a gel electrophoresis (SDS-PAGE gel electrophoresis) to sort the proteins by its molecular weight size in individual protein bands. The separated protein bands are then transferred to a nitrocellulose transfer membrane. The proteins adhere to the membrane in the same pattern as they have been separated due to interactions of charges. To visualize the protein of interest the membrane is commonly first probed using a primary protein- specific antibody followed by a labeled secondary antibody. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. The bound antibodies are then detected by western blotting substrate (e.g. Pierce ECL Western Blotting Substrate), and then visualized by using adeveloping film (HyBlot CL Autoradiography Film) in the darkroom. The thickness of the band corresponds to the amount of protein present.

EXTERNAL LINK

https://mmpc.org/shared/document.aspx?id=316&docType=Protocol

MATERIALS

NAME	CATALOG #	VENDOR
SDS-PAGE Apparatus	1658003	Bio-rad Laboratories
Transfer Apparatus	161-0158	Bio-rad Laboratories
30% Acrylamide / Bis. solution	161-0158	Bio-rad Laboratories
Ammonium Persulfate	AC20153-0010	Sigma Aldrich
TEMED (Tetra Methylene Diamine)	161-0800	Bio-rad Laboratories
Bio Rad Precision Plus protein Standard	161-0374	Bio-rad Laboratories
Ponceau S solution	P-7170	Sigma Aldrich
Nitrocellulose Transfer Membrane	10600002	Amersham Biosciences
HyBlot CL Autoradiography Film	E3018	Denville Scientific Inc.
Pierce ECL Western Blotting Substrate	32106	Thermo Scientific
Medium Thick Blotting Paper (whatman paper)		Sigma Aldrich
Autoradiography Film Cassette	FBAC 810	Fisher Scientific
Sodium dodecyl sulfate (SDS)	BP166	Fisher Scientific
Bromophenol Blue	B392	Fisher Scientific
Tris Base	BP152	Fisher Scientific

NAME	CATALOG #	VENDOR
Sodium Chloride (NaCl)	S271	Fisher Scientific
Glycine	G46	Fisher Scientific
Glycerol	G789-3	Sigma Aldrich
2-Mercaptoethanol	BP176-100	Fisher Scientific
Triton X-100	AAA16046AP	Fisher Scientific
Primary antibody	Self-Interest	
HRP-conjugated Secondary antibody	Self-Interest	
Carnation non-fat dry milk	Self-Interest	

MATERIALS TEXT

Note:
Bio-Rad Laboratories RRID:SCR_008426
Sigma-Aldrich RRID:SCR_008988
Fisher Scientific, RRID:SCR_008452
Amersham Biosciences RRID:SCR_013566

Reagent Preparation:

Note: Use lab personal protections e.g. Safety glasses, gloves, lab coat during reagent preparations.

Reagent 1
10%APS: Dissolve 0.1g APS in 1ml ddH_2O in an Eppendorf tube, Vortex well to mix.

Reagent 2
10% SDS: Dissolve 10g SDS in 100 ml ddH_2O in a 200ml glass beaker. Cover the beaker with aluminum foil. Mix well with stir bar, label and store at room temperature.

Reagent 3
1.5 M Tirs-HCl (pH 8.8): Dissolve 90.86g Tris base (MW 121.14) in 400ml ddH_2O. Mix well with stir bar. Measure pH 8.8 with 6N HCl. Adjust final volume to 500ml with ddH_2O. Store at room temperature.

Reagent 4
0.5 M Tris-HCl (pH 6.8): Dissolve 30.28g Tris base (MW 121.14) in 400ml ddH_2O. Mix well with stir bar. Measure pH 6.8 with 6N HCl. Adjust final volume to 500ml with ddH_2O. Store at room temperature.

Reagent 5
10x Tris-Glycine SDS-PAGE Running buffer: Dissolve 30.3g Tris Base, 10g SDS, 144.2g Glycine in 800ml ddH_2O. Mix well with stir bar. Bring final volume up to 1000 ml. Working concentration 1X.

Reagent 6
10X TBS (pH 7.5): Dissolve 12.1g Tris Base, 87.6g NaCl in 900ml ddH_2O. Mix well with stir bar, then pH to 7.5 with concentrated HCL. Bring final volume up to 1000 ml.

Reagent 7
1xTBST (wash buffer): Combine 100ml of 10x TBS, 900ml ddH_2O and 0.5-1ml TWEEN-20 (0.05-0.1% Tween-20).

Reagent 8
10xTransfer buffer: Dissolve 24g Tris Base, 115.2g Glycine in 900ml ddH_2O. Mix well with stir bar. Bring final volume up to 1000 ml. Store at room temperature.

Reagent 9

1x Transfer buffer: Combine 100ml of 10x Transfer buffer, 200ml Methanol, 900ml ddH_2O. Mix well. Store at 4ºC.

Reagent 10

5% Carnation non-fat milk in 1x TBST: Combine 5g dry milk, 10 ml of 10X TBS, 90 ml ddH_2O, 0.1 ml TWEEN-20 (or 1ml of 10% TWEEN-20). Always make fresh milk or 5% milk can be store at 4ºC for 24 hours.

Reagent 11

6x SDS Sample buffer for 10ml (protein loading dye):

- ddH_2O 2.875ml
- 0.5M Tris, pH 6.8 1.875ml
- Glycerol 1.5ml
- 10% SDS 3.0ml
- 2-β mercaptoethanol 0.75ml (use under the flow hood)
- 0.05% Bromophenol Blue 5mg or a trace of powder with the end of the spatula to make the desire color

Reagent 12

Separating Gel Preparation for 2 gels (Lower gel):

	10% gel	12% gel	15% gel
Millipore-Water	7.9 ml	6.56 ml	4.6 ml
30% Acrylamide/Bis solution	6.7 ml	8.04 ml	10 ml
1.5M Tris-HCL, pH 8.8	5.0 ml	5.0 ml	5.0 ml
10% (w/v) SDS	200 ul	200 ul	200 ul
10% (w/v) APS	200 ul	200 ul	200 ul
TEMED	10 ul	10 ul	10 ul

Add all reagents in a 50 ml conical tube except TEMED, vortex to mix, and then add TEMED into the mix just before use, invert tube gently couple of times to mix.

Reagent 13

4% Stacking Gel Preparation for 2 gels (Upper gel): *Note: 10% AP and TEMED solidify the solution quickly therefore add these two just before use.*

Millipore-Water	5.6 ml
30% Acrylamide/Bis solution	1.7 ml
0.5M Tris, pH 6.8	2.5 ml
10% (w/v) SDS	100 ul
10% (w/v) APS	100 ul
Temed	10 1

1) 6x sample buffer (protein loading dye):

- ddH_2O ---------- 2.875ml
- 0.5M Tris, pH 6.8 ---------- 1.875ml
- Glycerol ---------- 1.5ml
- 10% SDS ---------- 3.0ml
- 2-β mercaptoethanol ---------- 0.75ml (use under the flow hood)
- 0.05% Bromophenol Blue ---- A trace of powder with the end of the spatula to make the desire color

1 Wash the plates and wipe the surface with ethanol and dry plates.

2 Assemble casting frames and glass plate. Put small glass plate on the top of big glass plate and fit whole thing into the casting stand with small plate outside. Close casting frame door, fill the space between two glass plates with H_2O to check for leaks. Mark a small line at 2cm from the top of the glass with sharpie.

3 Pour off water and wipe dry with Kim wipe or tilt the apparatus, insert a thin strip of Whatmam filter paper along the side to dab all the remaining water from bottom.

4 Make separating gel (the higher the molecular weight of the protein, the smaller the percentage of the gel) in a 50ml conical tube. Mix the gel components by inverting the tube 6 times gently. Pour gel with a 5ml pipette or transfer pipette right in the middle of the plates up to the line drawn 2cm from the top of the glass. Add 1ml of 2- propanol (or water saturated n-Butanol at ratio of 1:10, or absolute Ethanol) on the top of the running gel to level the gel and remove bubbles. Wait about 40 minutes or until gel is polymerized.

5 After complete polymerization, pour the 2-propanol off and gently rinse the top of the gel with ddH_2O. Carefully blot off excess water with a thin strip of Whatmam filter paper **(DO NOT TOUCH THE GEL).**

6 Make 4% stacking gel, pour stacking gel on top of the running gel and insert the comb (s), making sure there are no bubbles under the teeth of comb. Wipe off any stacking gel that ran over the side of the gel sandwich. Wait until gel is polymerized. While the gel polymerizes prepare samples and make 1x running buffer from 10 x stocks.
Note: *4-12% pre cast gradient gel can be used to separate protein of all sizes very well and omits steps 1-6 (Novex Wedge well, 4-12% Tris-Glycine gel, 1.0mm x 15 well, Invitrogen, cat# XP04 125 BOX quantity 10/box).*

7 Thaw samples on ice.

8 Boil about 500 ml water in a 1000 ml size Pyrex glass beaker on Hotplate for boiling samples.

9 Label new screw top tubes for each sample.

10 Add 10-50 µg of each protein sample or as needed into the respective labeled tube.

11 Add 1/5 of protein volume of the 6x sample buffer (Protein: Sample buffer ratio 5:1) to each sample and then adjust the desire final volume with ddH_2O.

12 Boil samples at 95ºC for 5minutes. Do not need to boil Bio Rad Precision Plus protein Dual color Standard markers. Cool samples at room temperature and quick spin down.

13 Place gel inside the electrophoresis tank with small plate inside, press down and close doors.

14 Pour the running buffer into the electrophoresis tank (fill inside of chamber with 1x running buffer to about 1⁄2 way between top of small and large plate, then pour more running buffer into outside and to the bottom of the gel apparatus. Gently remove the comb. Rinse each well carefully with running buffer from the tank to flush out broken gel pieces from each well (use P-200 pipette to rinse)

15 Load protein marker (5µl), protein samples and + & - controls. Put lid and connect to a power supply (When connecting to the power source always connect red to red, Put lid and connect to and black to black). Run the gel with higher voltage (140 V) for stacking gel and use low voltage (100V) for separating gel.

16 Run the gel for approximately 1-1/2 hour, or until the dye front runs off the bottom of the gel (depending on the size of the protein).

17 Separate glass plates carefully and retrieve the gel, remove the stacking gel from the top of the gel and soak gel in transfer buffer for 15 minutes.

18 Cut and label nitrocellulose to the size of gel.
Note: *You can write at the upper right corner of the membrane with a pencil for date, µg of protein loaded, gel %, 1^0 and 2^0 antibody dilution, exposure time etc.* ***DO NOT WRITE IN THE MIDDLE OR PROTEIN CONTAINING AREA OF THE MEMBRANE.***

19 Soak nitrocellulose membrane in distilled water for 5minutes and then in transfer buffer for about 10-15minutes. In another dish, soak two fiber pads and two medium- thick whatman filter paper for each gel.

20 Pour some transfer buffer in a baking dish and assemble the sandwich with black side of gel holder cassette down in transfer buffer. Assemble the sandwich following way (make sure there are no bubbles between each layer)-

a) Put 1 wet fiber pad on the top of the black side of gel holder cassette

b) 1Whitman filter paper on the top of fiber pad

c) Gel on the top of Whitman filter paper

d) Nitrocellulose membrane on the top of the gel

e) 1 Whitman filter paper. Remove bubbles gently and carefully by rolling out a 10ml pipette over the Whitman paper

f) 1 fiber pad over Whitman filter paper.

Make sure there are no air bubbles between the gel and membrane, and squeeze out extra liquid and secure the sandwich inside the cassette by sliding the plastic clip of cassette.

21 Put a small magnetic stir bar in the bottom of the transferring tank and place the sandwich in the transfer unit with the black part in the back to match with black. (Protein runs from black to red, to the membrane).

22 Place electrodes on top of the sandwich. (When connecting to the power source always connect red to red, and black to black). Put a block of ice pack inside the apparatus. Fill the unit with 1X transfer buffer until the sandwich is covered with the buffer.

23 Transfer at 0.25Amp constant current for 2-3 hours-depending on size of protein (bigger protein needs longer run) or transfer overnight in a cold room at a constant current of 10-40mA.

24 After transfer protein from gel to membrane, remove blot from the transfer apparatus, briefly rinse the blot in water and then stain the blot with enough Ponceau S solution to check the transfer quality and also to see whether equal amounts of protein were loaded. Shake membrane in Ponceau S on a shaker until bands are visible, then wash the blot quickly with 1x TBST. Wrap blot in a saran wrap, make sure there are no wrinkles over the saran wrap, and make a photocopy.

25 Wash the membrane in 1X TBST 5-6 times for 5 minutes each wash or until all bands are gone.
Note: *All washing steps should be done with 1X TBS+0.1% Tween 20 (1xTBST) on a shaker at room temperature to ensure even agitation.*

26 Block the membrane: Cut a plastic bag (a seal meal bag) to a size so that membrane can be inserted easily. Put membrane inside the bag carefully. Add 10ml of 5% non- fat milk in 1xTBST in the bag. Remove all bubbles from the bag and seal the bag with an electric sealer. Incubate for 2 hours at room temperature or overnight at 4ºC on a shaker.

27 Dilute primary antibody (according to manufacturer instructions) with 10ml of 5% non-fat milk in 1x TBST. Vortex to mix. Remove blocking milk from the bag containing membrane, add 10ml diluted antibody into the bag, remove bubbles, seal the bag, incubate for 2 hours at room temperature (turn membrane over every 10-20 minutes) or overnight at 4ºC on a shaker (Primary antibody can be re-used).

28 Wash membrane 3 times for 5 minutes each with 1xTBST.

29 Dilute secondary antibody with 10ml of 5% non-fat milk in 1xTBST. Add 10ml of diluted secondary antibody into a new seal meal bag, put membrane in the bag after wash, remove bubbles from the bag, seal the bag and incubate for 1 hour at room temperature on shaker (turn membrane over every 10-15 minutes).

30 Quick rinse once with 1x TBST, then wash 4-6 times for 5 minutes each with 1xTBST.

31 Wash in 1X TBS for 5-10 minutes.

32 Develop blotting membrane with Western Blotting Substrate:
i. Place a piece of saran wrap over a clean glass plate. Remove all wrinkles from the surface of the saran wrap.
ii. Prepare ECL mix in a 15 mL conical tube 1:1 mixture of solution 1 and solution 2 (need 4ml per gel). Mix by pipetting up and down.
iii. Add ECL mix all over the membrane, let sit for 5 minutes at room temperature (use a 1000µl pipette to ensure that ECL covers the top andbottom of the membrane).

33 Place another piece of saran wrap over a clean-dry glass plate. Remove all wrinkles from the surface of the saran wrap. Dab the corner of the membrane on a paper towel to blot off excess ECL and place the membrane over the saran wrap carefully. Place the membrane on the with protein side facing down. Wrap the membrane nicely with extra hanging portion of the same saran wrap. Trim excess saran wrap and make sure there are no wrinkles or bubbles trapped on the protein side of the wrap (plastic sheet protector can be used to wrap blot).

34 Place the wrapped membrane in a film cassette with the protein side facing up. Secure the membrane to the cassette with small pieces of scotch tape.

35 Take cassette, film, timer and a marker to the dark room. Set up timer for 30 seconds and 2 minutes to start. Turn off all lights except those are safe for X-ray film exposure (red safelight).
Note: *Use gloves during the entire film-handling process.*

36 Carefully place film on top of the membrane and exposed for 30 seconds to start, turn on timer immediately. Quickly bend the upper right corner of the film to identify the position of the film inside the cassette. Take the film out of the cassette and place at the entrance to feed it into the developing machine. Press the 'start'button of the machine and film will go inside the machine itself (lightly touch theback edge of film before press 'Start' button to make sure it is completely inside).Wait until the film is developed. Adjust the exposure time from there according to the intensity of the band and the background.
Note: *Light emission is most intense during the first 5-30 minutes after substrate incubation. Light emission continues for several hours but decreases with time. Longer exposure times may be necessary as time elapses.*

37 After develop, put the film back in exact position over the membrane of the cassette (bent corner of the film will be placed into the upper right corner of the cassette) and then trace the protein marker bands from membrane on the film with a red marker to compare and determine the sample protein.