In-Person Western Blotting Lab Manual Instructions

Western blotting

Learning goals:

To understand the theory behind Western blotting experiments
To perform Western blots and interpret the results

Background:

In the technique of **Western blotting**, proteins in a sample are separated using SDS-PAGE (see below) and transferred to a membrane. Unbound sites on the membrane are blocked with a generic protein solution, and primary antibodies are applied to the membrane and allowed to bind. After the primary binding period, unbound antibody is washed away. A secondary antibody conjugated to an enzyme is then applied and allowed to bind, and the excess is again washed away. Substrate for the enzyme is applied to the membrane, and where the antigen was present, if primary and secondary antibody bound successfully, a colored product will be deposited over the antigen band.

Protein concentrations can be determined using the **Bradford assay**. In this assay, an acidic solution of Coomassie Brilliant Blue G-250 dye will change its spectrophotometric absorbance maximum from 465 nm to 595 nm if it binds to protein. Using a standard protein solution of known concentration and creating a standard curve for dilutions of the known solution, the protein concentrations of unknown solutions can be calculated.

SDS-PAGE stands for **sodium dodecyl sulfate-polyacrylamide gel electrophoresis**. SDS-PAGE separates proteins according to their molecular weight. Proteins are unfolded by the addition of reducing agents such as dithiothreitol or 2-mercaptoethanol that reduce disulfide bonds. The SDS coats the proteins and gives them a uniform charge to mass ratio. Molecular weights of proteins on SDS-PAGE gels can be calculated by plotting the logarithm of protein molecular weight versus the relative mobility (distance migrated into the gel), and by comparing the migration of the unknown protein sample to the migration of known molecular weight marker proteins run in a separate lane of the gel.

Using an alkaline phosphatase-conjugated secondary antibody and bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate, the sensitivity of the Western blot procedure is in the 100 pg range—VERY sensitive. Western blotting is the technique of choice for proving HIV infection after an initial ELISA assay screen, and for quantitating viral loads in patients. The BCIP/NBT is converted to a black-purple precipitate by alkaline phosphatase activity.

Tubulin is the major component of microtubules, a key structural element in almost all eukaryotic cells. Microtubules function during mitosis, intracellular transport, flagellar movement, and in the cytoskeleton. Tubulin is a heterodimer of two different 55 kDa subunits. We will be using a monoclonal anti-□-tubulin antibody of IgG1 isotype, and a goat anti-mouse IgG-specific alkaline phosphatase-conjugated secondary antibody, with NBT/BCIP substrate, to compare the tubulins in bovine and chicken tissues.

Procedure:

A. Prepare the Samples (day 1)
B. Determine the protein concentration (day 1)
C. Set up and run the gel (day 2)
D. Remove the gel (day 2)
E. Set up the protein transfer (day 2)
F. Immunoblotting: Block, Primary and Secondary Antibody (day 3)
G. Substrate preparation and application (day 3)

A. Prepare the samples:

Tissue Lysis buffer: 2% sodium dodecyl sulfate (SDS)
100 mM dithiothreitol (DTT)
60 mM Tris pH 6.8
NO bromophenol blue!
in deionized water

1. Weigh an empty 1.5 ml Eppendorf tube

2. Place about 0.2 g of tissue into the Eppendorf tube

3. Add 0.5 ml of tissue lysis buffer; Mix vigorously by vortexing

4. Place the sample in a boiling water bath for 5 minutes

5. Centrifuge the samples at 14,000 rpm for 10 minutes in the Eppendorf centrifuge at 4°C (in the cold room)

6. Remove the supernatant to a new clean tube in an ice bucket. Label each tube with your initials and the type of tissue you extracted and the date. These samples are your crude lysates.

B. Determine the protein concentration:

1. Prepare dilutions of the protein concentration standard (the New England Biolabs Bovine Serum Albumin reagent diluted to 1 ug/ul works well as a standard).
Set up 6 spectrophotometer tubes for BSA standards; label them 0,50,100,250,500, 750
Note: These numbers indicate concentrations, in ug/ml
Add 40 ul of BSA standard and water to a total of 1600 ul

Note: the linear range of the Bradford assay is 1.2 to 10.0 ug/ml

2. Sometimes the protein concentration in the crude lysate is so high that it exceeds the limit of accurate measurement of the spectrophotometer. To get around this problem, you will prepare two different dilutions of your crude lysate. At least one of these will be within the range of the spectrophotometer.

Prepare 2 dilutions of each of your crude protein lysates in separate glass spec tubes:

Dilution 1: Mix 4 ul of your lysate with 1596 ul of water in one glass spec tube

Dilution 2: Put 6 ul of your lysate into a plastic 1.5 ml Eppendorf tube with 18 ul water; mix Remove 4 ul of this 1:4 dilution into a new spec tube
Add 1596 ul of water to the 4 ul of diluted lysate in the spec tube

Did you do this for each of your crude lysates?

3. Add 0.4 ml of the BioRad Protein Assay Reagent to each Tissue Extract Dilution spec tube and each BSA Standard spec tube; mix by vortexing.

4. Incubate at room temperature for least 5 minutes but not more than 1 hour before reading Absorbance.

5. Prepare the digital spectrophotometer and zero it against water:
Turn it on and warm it up at least 15-20 minutes before using
Set the wavelength for 595 nm
With no tube in the spec, press calibration button and wait
Make sure the instrument changes to absorbance mode before using it on samples

6. Read the absorbance at 595 nm wavelength in the spectrophotometer for each sample.

7. Construct a standard curve graph for your BSA dilutions. Plot the concentration of BSA added on the x axis, and the absorbance on the y axis, on regular graph paper.

8. Locate your unknown protein samples' absorbance on your standard curve and extrapolate the protein concentration in your dilutions by dropping a line down to the X axis to read the concentration of protein in your dilution.

9. Calculate the protein concentration in your samples based on the dilution factors.

Protein Concentration in lysate=
(concentration from your standard curve) x (reciprocal of your dilution factors)

10. Label each concentrated lysate tube in the ice bucket with the protein concentration in ug/ml.

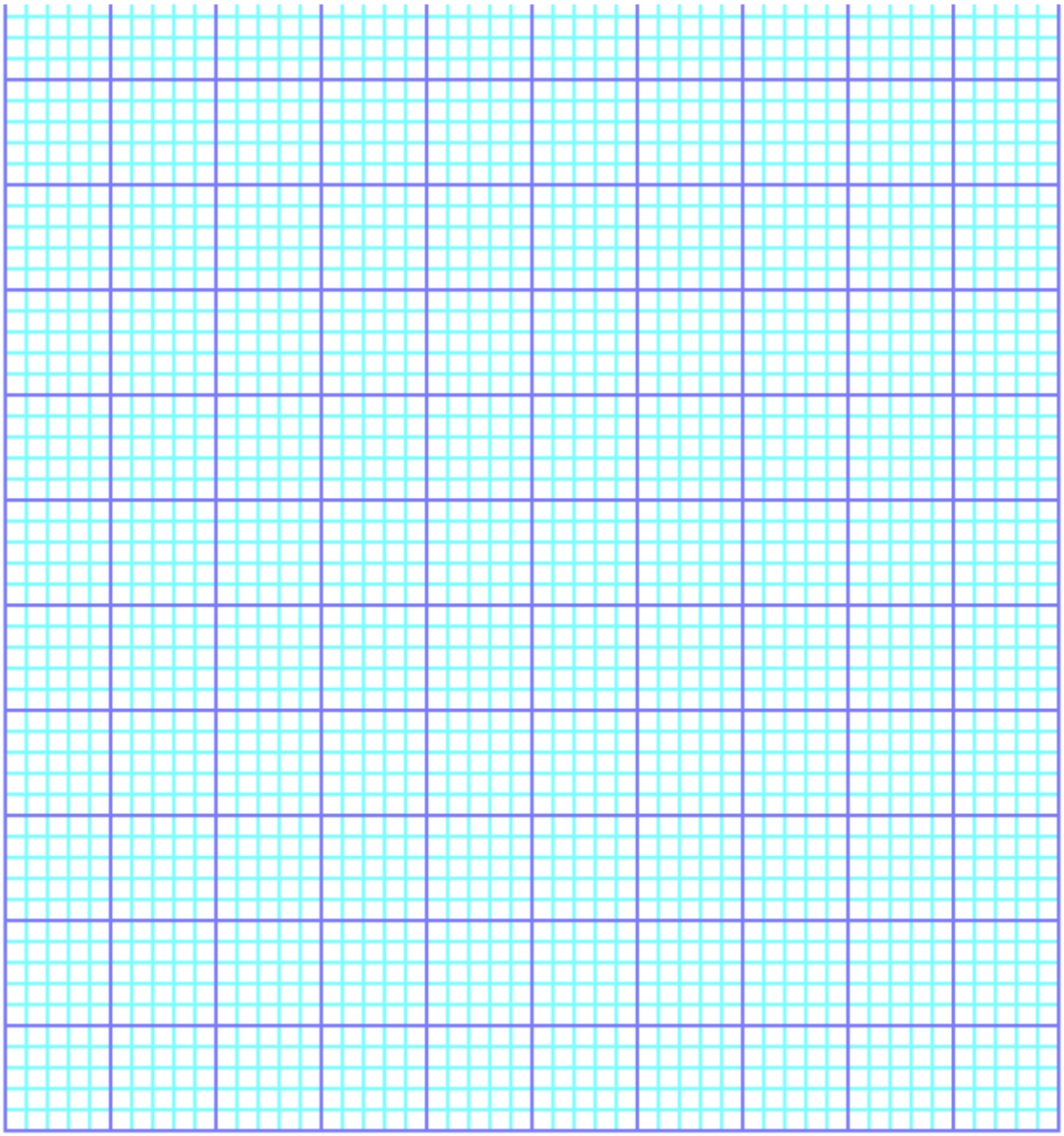
11. From these data, calculate the volume of your lysates you will need to give 10, 20, or 50 ug of protein loaded per gel lane next week, depending on the Professor's instructions.

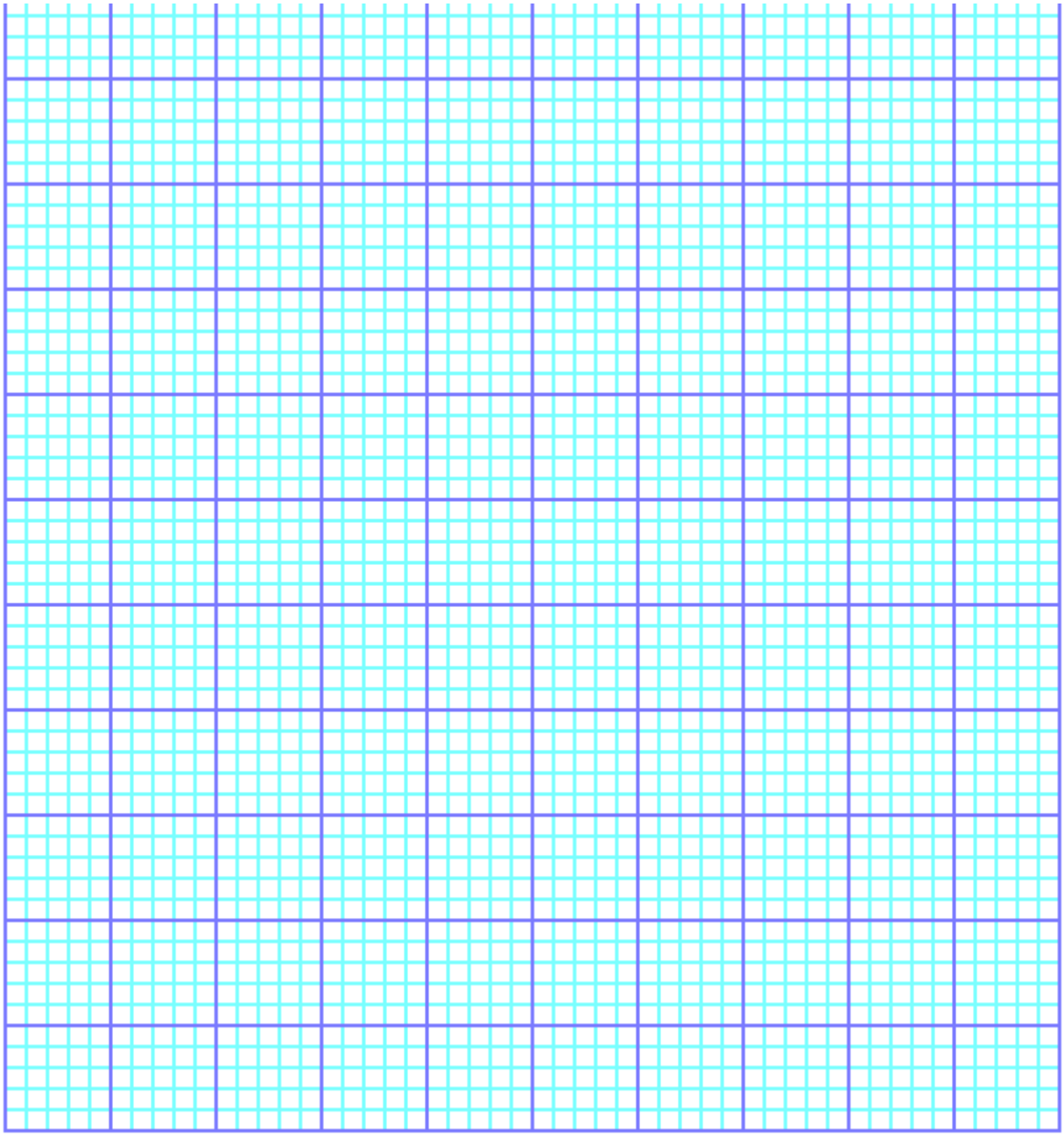
12. If you need to prepare further dilutions of your lysate to get 10-50 ug in an approximately 10 ul volume, do it!

13. Aliquot the necessary volumes of your crude protein extracts into labeled 1.5 ml screw-cap tubes.

Add one-half volume of 3X SDS buffer with DTE (e.g., if you needed 10 ul of your protein extract, add 5 ul of 3X SDS buffer).

14. Store your aliquots and your crude extracts in the minus 20 or minus 80 freezers until next week's lab period.





C. Set up and run the SDS-PAGE (usually done on Day 2 of the experiment)

Running buffer:
25 mM Tris
192 mM glycine
0.1% SDS
pH should be 8.3—do not adjust it!

Sample buffer—working concentration:
62.5 mM Tris-HCl, pH 6.8
2% SDS—coats the proteins to give them uniform charge:mass ratio
25% glycerol—increases sample density to make gel loading easier
0.01% bromophenol blue—dye to help visualize samples while loading
5% 2-mercaptoethanol— or 350 mM DTT (added fresh)-reduces disulfide bonds to unfold proteins

1. Thaw your aliquoted samples from last week in an ice bucket.

2. Don't forget to plan for Kaleidoscope Protein Molecular Weight Markers too, in their own gel lane—use 5 ul of the markers (composition of the markers is given below).

Kaleidoscope Protein Molecular Weight standards (Bio-Rad Catalog #161-0375):

Protein Identity	Color of Band on Gel	Molecular Weight (Daltons)
myosin	blue	250
beta-galactosidase	magenta	150
bovine serum albumen	green	100
carbonic anhydrase	violet	75
soybean trypsin inhibitor	orange	50
lysozyme	red	37
aprotinin	blue	25

Alternatively, you may use standards with molecular weights of 250 (blue), 150 (purple), 100 (blue), 75 (red), 50 (blue), 37 (green), 25 (red), 20 (blue), 15 (blue), and 10 (yellow) kd, depending on reagent availability

3. Heat the diluted samples at 95°C for 5 minutes in a temp block before loading
This will help to unfold the proteins and break up any intermolecular interactions

4. Put the sample into an ice bucket immediately after heating

The following sections 5-16 are modified from the BioRad Ready Gel Applications bulletin:
Note: wear gloves when handling the gels to avoid contaminating them with human proteins, and to protect your hands and avoid staining your skin and clothing.

5. Do not remove the 4-15% Bio-Rad Ready Gel from the pouch until you are ready to use it
Note: if only 4-12% gels are available, these will also work.

6. Remove the tape at the bottom of the gel along the black line.

7. Gently remove the plastic comb from between the plates by pushing it upward with your fingertips.

8. Place the gel cassette in the electrode assembly with the shorter plate facing the middle of the assembly, and the longer plate facing outwards. When placed correctly, the gel will rest at a 45° angle against the assembly. Push the gel up into place to form a tight, leakproof seal.

9. After both gels or one gel and the buffer dam are in place, put the assembled electrode assembly into the clamping frame.

10. Using both index fingers, push down gently on the electrode assembly to seat in place. At the same time, use your thumbs to close the clamping frame's cam levers and lock the gels in place. Place the electrode assembly into the gel tank. Test to be sure the lid fits on before completing the next step.

11. Fill the upper buffer chamber with about 125 ml of running buffer. Check for leakage. Reseat if leakage is too fast.

12. Fill the lower buffer tank with 200 ml of running buffer to the marked line

13. For best results, gently use a pipette and 1X tris-glycine SDS (TGS) buffer to rinse any debris out of the wells before loading

14. Load samples using a Hamilton syringe or a pipet with gel loading tips. Make sure you keep track of which sample is in which lane!

15. Place the lid on the tank, aligning the color-coded banana plugs and jacks.

16. Run the gel at a constant 200 Volts for 35-50 minutes, or for the time your Professor indicates to you—until the Kaleidoscope standards are well separated.
the starting current should be about 50 mA/gel
the finishing current should be about 30 mA/gel

D. Removing the gel:
Note: wear gloves when handling the gels to avoid contaminating them with human proteins, and to protect your hands and avoid staining your skin and clothing.

1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.

2. Remove the tank lid and carefully lift out the inner chamber assembly. Pour off the running buffer.

3. Open the cams of the clamping frame. Pull the electrode assembly out of the clamping frame and remove the gel cassettes.

4. Remove the gels from the gel cassette by gently separating the two plates of the gel cassette using the green metal tool that came with the gels.

5. Be sure to clip one corner (usually the lower left corner) of the gel so you can tell its orientation later on. Write down which corner you clipped here:_____

6. Remove the gel by floating it off of the glass plate: invert the gel and plate under gel transfer solution (see below), agitating gently until the gel separates from the plate.

7. Using a razor blade, cut off the stacking gel and bottom gel plugs if present to leave a uniform thickness of gel

E. Setting up the protein transfer onto nitrocellulose or PVDF membranes:

Transfer buffer (good for proteins less than 80,000 molecular weight):

component	concentration	for 1000 mL
Tris	2.5 mM	Use 10X concentrate
Glycine	19.0 mM	Use 10X concentrate
Methanol	20%	200 mL
Distilled water		to 1000 mL total volume

1. Chill the transfer buffer in a container in an ice bucket

2. Equilibrate the gel in transfer buffer for 15 minutes on a rocking or shaking platform.

3. Soak the transfer apparatus fiber pads thoroughly in transfer buffer in tupperware.

4. **NOTE: Use gloves when handling Western blot membranes to avoid inoculating them with human proteins**
Mark the membrane with initials and orienting marks in pencil, then:
<u>If using nitrocellulose membrane:</u>
prewet it in transfer buffer along with the blotting paper.
OR
<u>If using PVDF (nylon) membrane:</u>
Soak the PVDF membrane in 100% methanol for 1 minute first
Then soak the membrane in distilled water rinses, 3 times for a 2 minutes each
Then soak the membrane in transfer buffer

5. Pour about half of the transfer buffer into a Tupperware bin big enough to hold the blotting sandwich apparatus

6. Peel gel off plate into transfer buffer in Tupperware

7. Assemble the gel transfer “sandwich” IN THIS ORDER:
KEEP EVERYTHING SUBMERGED/AS WET AS POSSIBLE

ROLL OUT AIR BUBBLES WITH A GLASS PIPETTE AS YOU ADD EACH LAYER

a. plastic plate with black side down—this is the bottom of your sandwich
b. sponge or fiber pad presoaked in transfer buffer
c. whatman 3 mm paper presoaked in transfer buffer
d. gel—roll out any bubbles with a Pasteur pipette
e. presoaked transfer membrane—roll out any bubbles again
f. whatman 3mm paper sheet presoaked in transfer buffer
g. sponge or fiber pad presoaked in transfer buffer
h. plastic plate with clear side facing up—this is the top of your sandwich, to be placed closest to the positive electrode (red anode) in the apparatus

8. Insert the sandwich into the transfer apparatus with the transfer membrane closest to the positive electrode (red anode). Make sure you put the little "feet" facing down to leave room for the small stir bar to be placed underneath it.

9. Add a frozen Bio-ice module to the tank.

10. Fill the tank with transfer buffer. Insert the tiny "flea" stir bar beneath the gels.

11. Run the transfer 2.5 hours-overnight at 20-25 volts/93 mA in the cold room.

12. Turn off the power and carefully disassemble the sandwich, **being sure to mark the location of the wells and the marker bands with a PENCIL (the alcohol will dissolve marks made in pen)**

13. If several students' samples are on a single membrane, cut the membrane apart, being certain to mark it so you can orient it later on.

14. Rinse the blot in 1X PBS briefly, then blot dry on Whatman paper.

15. Optional: may stain the transferred gel to make sure transfer was complete. Remember to soak out any SDS with several changes of water before adding Coomassie Blue.

16. To store your protein blots, put them on filter paper and seal them with Saran wrap. Store in a dry, dark place until next use.

F. Optional: Stain one gel or section of a gel with Coomassie Blue:

1 Transfer one plate with gel on it to a staining tray containing distilled water. Rinse the gel 3 times, 5 minutes each time, to remove SDS from the gels before staining. Residual SDS will cause a hazy background after staining.

2. After rinsing out the SDS, place the gel into Bio-Safe Coomassie stain. The staining liquid will cause the gel to detach from the plate. If not, the gel can be lifted gently from the plate up into the stain.

3. Stain the gels for 1 hour, with gentle agitation.
4. Destain the gels in distilled water 8 hours-overnight with changes of water.

5. Preserve the gels in Ziploc baggies for later observation and photography.

6. Coomassie-stained gels can still be used for Western transfer:
   a. re-soak the stained gel in 1X TGS running buffer with agitation for 30 minutes
   b. soak the gel in blotting buffer for 15 minutes; continue with Procedure E above.

G. Western blotting: Antibody incubations (usually done on Day 3 of the experiment)

Notes: 1X PBS is 1 mM sodium phosphate, 15 mM NaCl, pH 7.4
Blocking buffer is 1X PBS, 0.025% Tween20, and 5% nonfat dried milk
(0.02% sodium azide is optional)

Steps 1-4 will have been done for you by your Professor before lab.

1. Put the blot into an appropriately sized container (pipette box lids work nicely)
   Rinse it with PBS until all parts look thoroughly hydrated. This can take 30 minutes.

2. <u>Blocking solution:</u>
   Apply blocking solution to the blot: Use 10-15 ml/square Petri dish 1-2 hours at room temperature, on shaker platform.
   Alternatively, may use 5 ml blocking solution in a 15 ml tube for blots cut into strips.
   For a whole uncut gel's blot, will need 25 ml blocking solution.

3. Discard blocking solution.

4. <u>Primary Antibody binding:</u>
   Blocking solution with primary antibody diluted 1:250
   Primary antibody is Sigma anti-beta tubulin mouse monoclonal IgG1, #T4026
   Incubate for 1-3 hours room temperature, or overnight, on shaker platform

5. SAVE the primary antibody solution in its labeled tube for re-use by other labs.

6. <u>Wash</u>:
   Wash buffer is PBST: 1X PBS with 0.025% Tween20
   Wash blot 2 times, 5 minutes each time on rotator, 25 ml each wash
   Note: if binding in tubes—remove blot from tube, transfer to box lid for washes, and remember to wash out the tube thoroughly with tap water 3-4 times before next step.

7. <u>Wash</u>: with TBS: 150 mM NaCl/50 mM Tris pH 7.5
   2 times to get rid of PBS.

8. <u>Secondary Antibody binding:</u>

Tris-buffered saline (USE TBS, NOT PBS—THIS WOULD NOT GIVE OPTIMAL ACTIVITY OF ALKALINE PHOSPHATASE!) + 0.025% Tween20 + antibody-enzyme conjugate at final concentration of 5-0.5 ug/ml (check with Professor for appropriate dilution, usually 1:1000)

Incubate for 45 minutes room temperature on shaker platform

Note: in 2012, 1:1000 for AP-conjugated secondary antibody was probably too concentrated—try more dilute next time

Note: in 2015, class used Jackson goat anti-mouse IgG-peroxidase conjugate (AM lab at 1:10,000, PM lab at 1:1000) and Bio-Rad substrate kit, but the Westerns did not work.

Note: in 2018, used 1:1500 for AP-conjugated secondary antibody and it worked but had some background.

9. <u>Wash:</u> 2 times with 25 ml TBS + tween in original container 5 minutes each time
2 times with 25 ml TBS (no tween) 5 minutes each time
(even better--use larger volumes in larger container for last 2 washes)

10. Photograph the blot before the addition of substrate. You should see only the Kaleidoscope pre-stained protein molecular weight markers at this stage.

H. Preparation and application of substrate:

NBT stock solution: 0.5 grams NBT
10 ml 70% dimethylformamide

BCIP stock solution: 0.5 grams BCIP disodium salt
10 ml 100% dimethylformamide

Alkaline Phosphatase Buffer: 100 mM NaCl
5 mM MgCl2
100 mM Tris pH 9.5

Store all these stocks at 4°C until use

1. Just before you develop the blot, prepare fresh substrate solution:
Add 66 ul NBT stock to 10 ml of Alkaline Phosphatase Buffer; mix well
Add 33 ul of BCIP stock. Use within 1 hour.

2. Place the washed blot in a suitable container. Add 10 ml of substrate solution per 15 x 15 cm membrane. Develop the blot at room temperature with agitation until the bands are suitably dark. Typically, this takes between 3 and 30 minutes.

3. Stop the reaction: Rinse with PBS containing 20 mM EDTA to chelate Mg++ ions.

4. Photograph the results quickly—as the membrane dries out, the background will darken!

Review Questions:

1. How does the Bradford Assay work?

2. What is the purpose/function of the SDS in SDS-PAGE?
3. What is the purpose/function of beta-mercaptoethanol or dithiothreitol?

4. Why does one go through the process of transferring proteins from a gel on to a membrane for Western Blotting?

5. What is the purpose of the secondary antibody?

6. Why did the procedure switch from PBS to TBS at the end of the assay?

7. What information can a Western blot give that agglutination and precipitation and immunocytochemistry assays cannot give?

8. What similarities and differences, if any, did you observe between the bovine and chicken tubulins?

9. Prepare a graph of molecular weight versus migration for the Kaleidoscope protein markers. Then use that graph to extrapolate the molecular weight of the tubulin proteins in the beef and chicken samples.

10. Is the molecular weight of tubulin in your samples consistent with what is in the literature? If not—provide some possible explanations for any discrepancy.

References:

Harlow, E., Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Bio-Rad Protein Assay applications manual

Bio-Rad Ready Gels Application Guide

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J., 2004. Immunobiology: The immune system in health and disease, sixth ed. Garland Science, New York.