**ISOLATION OF PROTEIN FOR WESTERN BLOTTING AND PROTEIN QUANTITATION**

***Today we are going to isolate total protein from tissues isolated from two different growth conditions. These proteins will subsequently be used for a SDS/PAGE (Western) electrophoresis to estimate the level of RACK1A protein expression between these two samples. In order to make the extraction process to have biological meaning, we are going to compare the level of RACK1A protein expression in WT tissues that were collected under different growing conditions (TA will perform this part). To have more confidence in experiments where protein expression levels are compared, it is imperative that equal amount of total protein be loaded on electrophoresis gel. Therefore, we are also going to quantify the extracted protein concentration using BRADFORD protein assay.***

**INTRODUCTION:**

Proteins can be easily prepared for Western blotting by multiple cycles of freeze thawing, sonication, or lysis with enzymes and detergents. Mammalian cells are usually quite easy to lyse and generally do not require freeze thawing or the enzymatic treatments that are necessary to lyse yeast and certain bacterial cells. While one can lyse mammalian cells directly in the sample buffer used for SDS/PAGE electrophoresis, this buffer contains bromophenol blue, SDS and mercaptoethanol which at sufficient concentration can interfere with the commonly used Bradford protein assay. Hence it is best to lyse the cells in a buffer which contains low enough levels of these interfering species to allow accurate determination of the protein concentration. In many cases it has been found that merely omitting bromophenol blue prevent any interference with the protein dye binding assay. Once the cells are lysed, they should be boiled in a screw cap tube to denature any proteases (degrades protein) that may remain active in buffer. Today we are going to use a lysis buffer that allows efficient extraction of protein while avoiding protein degradation and interference with protein immunoreactivity and biological activity. The lysis buffer consists of a dialyzable mild detergent at a low concentration (for minimal interference with protein interactions and biological activity), bicine (a buffer preferable for biological activity), and 150 mM NaCl.

In order to estimate how much protein to expect in a sample one should consider the following. For mammalian cells one obtains about 1 g wet weight of cells from a liter of media. Assuming the cells have grown to about 106 cells/ml this gives 109 cells wet weight per gram. Since a cell is roughly 90% water and nearly everything else is protein, we can very roughly estimate that 109 cells contain 0.1 g of protein. So if 100 ml of cells are resuspended in 1 ml of sample buffer the concentration will be roughly 10 mg/ml. A volume of about 5 l or less of this protein extract is usually sufficient for determining the exact protein concentration.

PROCEDURE: *RACK1A* gene is known to involve in salt stress signaling pathway. However, it is not quite clear whether heat stress has any effect on the level of RACK1A protein expression. Therefore, we are going to isolate total proteins from +/- salt treated tissues from Wild Type plants. We will use rack1a knock out plants as control.

PROCEDURE: Before extraction of protein from supplied tissue powders, add 1X protease inhibitor solution to supplied lysis buffer. The lysis buffer is a non-ionic detergent-based reagent, which offers a convenient method for efficient plant cell lysis and protein solubilization. It's a non-denaturing reagent and maintains protein immunoreactivity and biological activity. The lysis buffer is efficient, rapid, and ready to use. It contains bicine buffer, which is preferable for many biological activities.

1. Add 2 ml of lysis buffer to 1 g of supplied tissue samples (if you are provided with 100 mg of powder, add 200 ul of lysis buffer). Mix well so that no powder clumps can be seen.

2. Incubate 10 minutes on ice.

3. Centrifuge the lysed cells for 10 minutes at 12,000-20,000*g* (4C)to pellet the cellular debris.

5. Transfer the protein-containing supernatant to a chilled centrifuge tube.

Note: Lysate preservation requires low temperatures. For long term storage it is recommended to store the lysate at –70 C.

**BRADFORD PROTEIN ASSAY (BIO-RAD REAGENTS) FOR PROTEIN CONCENTRATION MEASUREMENT**

**PROCEDURE:**

1. Dilute the dye with four volumes of D.I. water and filter out any remaining particulates (in case the concentration of dye is 4X). (This step will be done by the TA).

2. Add 5 l protein extract to 95 l with water in a glass test tube.

3. Add the ~100 l of diluted protein extract to 5 ml of diluted dye mix in a small test tube. Place Parafilm over the top of the test tube and invert several times to mix. If the mixture immediately turns dark blue start over and use less extract. Conversely if you are unable to detect by eye any difference between assay and blank add more extract.

4. Allow sample to develop for at least 5 minutes and measure at OD595 nm against blank and standard curve with same buffer and dye.

 Note: The Bradford reagent binds tightly to glass. This means that as you read consecutive samples the colored dye builds up on the cuvette walls and prevents and accurate reading of the sample OD. As we are using glass cuvettes, they must be rinsed out with ethanol between samples to remove the bound dye. An alternative approach would be to use disposable plastic cuvettes which are relatively inexpensive.

5. By comparison with a standard curve, estimate the Y g amount of protein in the X l added to the standard 5 ml reaction. Then convert the concentration estimate (Y g/X l) to g/l or g/ml.

STANDARD CURVE PREPARATION

**Protein Standards from 0 to 100 g per 5 ml reaction**

You should note from the discussion in the introduction to this protocol that the sample buffer contains a number of compounds that can interfere with the assay-in particular SDS. It is important not to exceed the allowed concentrations of these interfering substances in the assay. For SDS the maximum concentration compatible with the assay is 0.1% in a 100 l sample. (For a complete list of interfering substances see the BioRad manual**). In addition, as in any assay it is important that the standard curve contain exactly the same components in the same concentrations as the samples-except of course for the substance one is measuring.**

 With these considerations in mind, the following table gives instructions for constructing a standard curve for the Biorad protein assay. Prepare BSA stock solutions at concentrations of 2 mg/ ml in water.

 2 mg/ml stock (l) Lysis buffer H2O (l) Final gs of protein

 being added to 5 ml assay

 0 5 95 0

 5 5 90 10

 15 5 80 30

 25 5 70 50

 50 5 45 100

To construct the actual standard curve, add 100 l of each stock solution to a separate tube containing 5 ml of filtered protein reagent and read A595 after 5 min.

An example of the BSA standard curve will be constructed by your TA and the class will use the curve to deduce the unknown protein content.

**WESTERN BLOTTING: IDENTIFICATION OF SPECIFIC PROTEINS**

**INTRODUCTION:** **ISOLATION OF PROTEIN FOR WESTERN BLOTTING**

Western Blotting:

Western blotting is a method for identifying a specific protein in a complex mixture and simultaneously determining its molecular weight. The procedure can be broken down into a series of steps.

1. Size separation of the proteins in the mixture by Polyacrylamide Gel Electrophoresis (PAGE).

2. Transfer of the separated proteins to a membrane while retaining their relative position.

3. Detecting the protein under investigation by its specific reaction with an antibody and determination of its size relative to standard proteins of known size.

In practice there are usually a few intermediate steps consisting of gel and/or membrane staining. Gel staining is used to monitor whether proteins have separated properly on the gel and membrane staining is used to monitor the efficiency of the protein transfer to the membrane from the gel. Membrane staining is important to ensure that the results seen are not unduly influenced by the failure of proteins in particular area of the gel from transferring.

**Molecular Weight Markers**

 There are a number of different commercially available protein molecular weight markers which are used to estimate the molecular weight of proteins on Western blots. They generally fall into two categories, prestained and unstained. Prestained or dyed markers either have all the proteins in the molecular weight ladder stained with a single dye or alternatively each of the proteins in the ladder is stained with a different colored dye. The differently stained markers, while the most expensive, have the advantage of providing unambiguous identification of the specific proteins in the ladder. This prevents confusion as to which molecular weight marker band you are looking at which can sometimes occur when some of the fastest moving bands from standards dyed with a single dye run off the gel.

 **PROTOCOL**: **SEPARATION OF PROTEINS ON THE BASIS OF MOLECULAR WEIGHT: SDS GEL ELECTROPHORESIS**

**PROCEDURE:**

**Electrophoresis gel:** The manual that comes with your model electrophoresis unit will describe how to make your own gels using that specific equipment. The basic procedure, however, will be the same in most instances. First depending on the particular apparatus being used, you must assemble either two glass plates, or a single glass plate and notched alumina plate, separated by two plastic spacers which determine the thickness of the gel. Once the plates and spacers are assembled, the bottom has to be sealed with tape or agarose or clamped into a casting rig which prevents the gel assembly from leaking. Then you pour the dissolved gel up to a predetermined depth that is dependent on the depth of sample in the well. However, now a days various companies sell per-cast gels and depending on the size and nature of the proteins under investigations, you would buy the appropriate gels. For our purpose, we are using 12% Bis-Tris pre-cast gel which is suitable to separate 36 kD protein (RACK1) we are studying.

**Sample Loading and Final Assembly of the SE 250**

1. Open a pre-cast gel from its container and remove the white tape at the bottom to allow flow of electricity.

2. Mark the location of the wells using a marker pen and then gently remove the comb.

3. Rinse the sample wells with running buffer by pipetting up and down with Pasteur pipette.

4. Fill the sample wells and the upper and lower buffer chambers with running buffer.

5. To prepare samples: add 1X loading buffer (dye) to 50 g of protein. For example if your protein is dissolved in 30 l of buffer, then add 7.5 l of loading dye from a 4X concentration of dye. The loading dye will contain reducing agent beta- mercaptoethanol (5%).

6. Boil the samples for 5 minutes and then cool on ice.

7. Load the gel using appropriate tips. The tips should curve toward you as you load. Be careful to depress the Pipettman slowly as you load the well so as not to blow your sample out of the well.

8. Place the safety lid on the unit and attach the leads to the power supply.

**Running the gel**

9. The pre-cast gel we are using is recommended to be run for 2 hours at a constant voltage of 200 V.

10. When the tracking dye reaches the bottom of the gel (in about two hours), turn off the power supply, disconnect the leads, and remove the lid of the unit.

12. Lift gel sandwich off the core.

13. Use plastic gel wedges or gel knife to pry open the gel sandwich FROM THE BOTTOM and Sides. The gel will usually stick to the one of the plates. Gently peel the gel off the plate into a tray of water. If the gel sticks, use a wash bottle filled with water to help dislodge the gel while holding the gel just over the tray of water. The dislodged gel should slide into the tray. Stain gel or if desired, proceed with the Western transfer procedure.

**PROTOCOL: PROTEIN TRANSFER FROM ACRYLAMIDE GEL TO MEMBRANE**

**INTRODUCTION:**

There are two major methods used to transfer proteins electrophoretically to a membrane, semidry blotting and tank transfer blotting. We will be using tank transfer method.

**Tank Blotting**

This is a commonly used alternative to semidry blotting. Tank transfer uses much more buffer than the semidry procedure. The procedure for transferring proteins from gels to nitrocelluose filter is as follows.

1. You will be provided with a pre-cut nitrocellulose membrane.

2. Carefully wet the nitrocellulose membrane in transfer buffer. Start from one corner and slowly lay the filter in to the transfer buffer.

3. Soak the Whatman paper and nitrocellulose in transfer buffer for at least 5 min.

4. Fill a large glass tray with transfer buffer and place one of the transfer blotting pads in it.

5. Build a blot sandwich on the sponge: Whatman paper, gel, nitrocellulose, Whatman paper. Carefully layer each piece onto the sandwich. Do not get any bubbles in your sandwich.



6. Place sandwich into transfer tank. SDS in the denaturing gel gives proteins a large net negative charge. Place sandwich such that proteins will move out of the gel and onto the nitrocellulose. Nitrocellulose side of sandwich should face positive pole.

7. Transfer proteins for 90 minutes (two gels) at 120V with cooling.

8. Turn off power and remove blot and membrane.

**SOLUTIONS:**

**1. 10x Running buffer**

 Compound Amount/100 ml Amount/1000 ml

 Tris Base 3.0 g 30 g

 Glycine 14.4 g 144 g

1. H2O to 1 liter, do not pH

**2. Transfer Buffer**

 Final Concentration Stock Solution Amount/100 ml Amount/1000 ml

 0.1% SDS 0.1 g 1.0 g

 20 % methanol methanol 20 ml 200 ml

 1X Running buffer 80 ml 800 ml

**PROTOCOL: STAINING OF WESTERN BLOTS FOR TOTAL PROTEIN**

**INTRODUCTION:**

Many different procedures have been published for staining total protein on Western blots after transfer. Some employ dyes such as Coomassie Brilliant Blue R250, Amido Black, Ponceau S, Fast green FCF or Eosin Y. Others use colloidal dyes such as India ink or colloidal gold or iron. WE will use Ponceau S to stain our membrane.

Staining

1. First, prewet the membrane in PBS and submerge the prewetted blot for 30 sec in the staining solution. (If staining is not obvious, continue up to five minutes)

2. After proteins become visible on the membrane, rinse repeatedly with dIH2O to remove bulk of the stain from the protein bands.

3. Photograph the membrane. Lay a ruler beside the filter so that the location of the molecular weight standards can be readily determined if they fade during the hybridization process.

Destaining

1. Rinse repeatedly with deionized H2O to remove rest of the stains. Not all the dye is removed, but that which remains does not appear to interfere with subsequent antibody detection methods.

**PROTOCOL: RAPID IMMUNODETECTION PROTOCOL WITH ENHANCED CHEMILUMINESCENCE DETECTION (ECL)**

*SOURCE: ECL WESTERN BLOTTING PROTOCOLS, AMERSHAM CORP.*

This system is based on the detection of light emission from a secondary antibody linked to horseradish peroxidase which catalyzes the oxidation of luminol. Light emission is detected on x-ray film. Although this system is more expensive and less convenient than the colorimetric alkaline phosphatase detection system, the sensitivity can be incredible.

The rapid protocol presented here is essentially that presented in Appendix I of the Amersham technical book on ECL. If time is in short supply the following protocol allows the immunodetection using HRP-labeled antibodies to be completed in just over 2 hours, compared to 4 hours 15 minutes for the standard protocol. If desired, the protocol can be further shortened by also optimizing the primary antibody for a shortened incubation.

**PROCEDURE:**

1. Electrophoresis and blotting should be done according to your favorite protocol.

2. Blocking the membrane. Non-specific antibody binding sites on membranes must be blocked before probing to prevent high background and/or spurious binding to specific protein bands. Typical blocking reagents are 5% non-fat dry milk (blotto) or 10% BSA dissolved in phosphate buffered saline (PBS) or Tris buffered saline (TBS) solutions which contain 0.05% to 0.1% Tween 20.. Blocking may be accomplished in as little as 10 minutes or may require overnight at 4oC depending on the antigen/antibody background and the specific membrane being used. Both the blocking times and the optimal Tween-20 concentrations have to be determined empirically. We will use 5% non-fat dry milk dissolved in PBS with Tween-20 (PBS-T).

3. During the blocking stage dilute the primary antibody in PBS-T.

Note: The optimum dilution of the primary antibody will vary and should be determined for each antibody used. The RACK1 antibody is used at 1:1000 dilution.

4. Rinse the membrane 3 X for 5 minutes each in PBS-T. Slightly longer rinses are presumably acceptable.

5. Incubate the membrane in the diluted primary antibody for 1 hour (to overnight) at room temperature with agitation. We will use one hour of primary antibody incubation.

Note: A further shortening of the immunodetection is possible by increasing the primary antibody concentration. This allows a reduction in the incubation time without compromising sensitivity. However, the optimal increase, which saves time, but does not cause severe background problems, must be optimized for each new antibody.

6. Use PBS-T for the washing buffer. Briefly rinse the membrane twice using wash buffer to remove the bulk of the unbound antibody, then wash twice for 10 minutes with fresh changes of washing buffer at room temperature. Use as large a volume of wash buffer as possible at each 10 min. rinse to reduce background. As a guide, there should be at least 4 ml per cm2 of membrane present.

7. During the wash steps dilute the HRP-labeled secondary antibody in PBS-T (1:10,000).

8. Incubate the membrane in the diluted secondary antibody for 30 minutes at room temperature with agitation.

9. Wash the membrane as described in step 6.

10. Signal Generation/Detection

**Read through this whole section before proceeding. It is necessary to work quickly once the blots have been exposed to the detection solutions.** All steps can be carried out in a dark room; it is only necessary to switch off the light after step J below. Equipment that is needed includes an X-ray film cassette, a roll of Saran Wrap (other 'cling-films' may not be suitable), a timer and blue-light sensitive autoradiography film, for example Hyperfilm-ECL (RPN 2103). If possible, wear powder-free gloves because powder spots cause blank areas on films.

a. Mix an equal volume of detection solution 1 with detection solution 2 to sufficiently cover the blot, in this case 5 ml of solution 1 and 2.

b. Place a large piece of Saran Wrap on flat bench. Use clean Kimwipe to smooth out the Saran Wrap so that it is perfectly flat.

c. Use forceps to remove the blot. Be careful to only clamp the edges of the blot to prevent crushing the lane areas. Crushing sometimes causes high background.

d. Remove excess wash buffer by holding the blot vertically and touching one of the lower corners to a small pile of Kimwipes. Wait until all the excess moisture is wicked from the blot.

e. Place the blot in the middle of a sheet of Saran Wrap with the protein side up.

1. Using pipette draw up mixed developing solution and apply the solution to the corners and middle of the blot. Add enough so that the entire blot is covered and you can see a positive meniscus. Surface tension will hold the fluid on top. Stop adding the solution if it begins to flow out on the surrounding page protector or Saran Wrap.

Alternatively, if one is processing a number of blots, you and just fill the bottom of a large Petri dish with substrate and immerse the blots in this solution.

1. Incubate for 1 minute at room temperature.
2. After 1 min., remove the blot from the substrate, and place the filter on the lower sheet of the plastic sheet protector. Close the sheet protector gently. Wipe back and forth with several clean Kimwipes to remove the excess liquid and any bubbles that form. Be sure to go around the edges and dry off the excess developing reagent. If the cover is damp it will cause the film to develop improperly.
3. Turn on the safe light and close the door. Place the sheet protector and membrane in a film cassette, then take out a piece of film, immediately apply the film on top of the sheet protector, and close the cassette cover and expose the film 5 minutes to several hours depending on the strength of the signal.
4. Develop the film by placing it in the developer tank for 90 seconds, rinsing it in the water tank for 90 seconds, fixing it in the fixer tank for 90 seconds then placing it in the water tank for 2 min., followed by drying it in the dryer. If the exposure is too light or too dark, adjust the exposure time accordingly. As soon as possible, leave the darkroom, so that it will be available for others.

k. Once the autoradiograph is developed, align the film relative to the filter using

 the phosphorescent markers as a guide and mark on the autoradiographic film

 where all the markers fall. Use the distance as a guide to determine the

 molecular weight of the unknown protein band on the film.