

## Week 1: Protein isolation and quantification

### Objective

The objective of this lab exercise is to obtain protein samples from fruit fly larvae, BCS and FBS, all of which are then quantitated in the preparation for next week's SDS-PAGE and Western blot.

### Surface sterilization of larvae

1. Obtain a culture of *Drosophila* larvae. The larvae should be ~5 days old (third instar). These are the largest soft ones often found crawling above the media on the glass (not the pupae).
2. Select thirty-five larvae for the following protocol and transfer them into the well of a spot plate containing Ephrussi-Beadle Ringer's (EBR) solution (0.129 M NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 35 mM Tris.HCl pH 6.85).
3. Gently wash the larvae about 1 min., then transfer them to a second well containing 70% ethanol (EtOH). Wash in EtOH about 1 min and then transfer them to a third well containing EBR.

### Preparation of protein samples for SDS-PAGE

1. Transfer the larvae to a pre-chilled 1.5 ml microcentrifuge tube.
2. Add 200 µl cold EBR and rinse the larvae to the bottom.
3. Homogenize gently but thoroughly and then add another 100 µl cold EBR.
4. Centrifuge the tube for 1 minute at top speed in the microcentrifuge.
5. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet. (Keep the protein containing tube on ice to prevent protein degradation.)
6. Transfer a 100 µl aliquot from the tube to a new PCR tube and put on the ice (Keep the rest of sample on ice.)
7. Prepare **bovine calf serum** (BCS) or **fetal bovine serum** (FBS) samples. Dilute each individually with distilled water to make 150 µl of 10 %, 1% and 0.1% in three separate microcentrifuge tubes. **Mix well before each dilution!**
8. Take 100 µl aliquots from 0.1% and 1% BCS or FBS samples into two new PCR tubes and put on the ice.
9. Add 50 µl 3X SDS-Laemmli sample buffer (187.5 mM Tris pH 6.8, 6% sodium dodecylsulfate (SDS), 30% glycerol, 15% β-mercaptoethanol (β-ME), 0.015% bromophenol blue(BPB)- **Mix well before each pipetting**) to all three tubes containing 100 µl aliquots of *Drosophila*, 0.1% and 1% BCS/FBS samples.
10. **Mix well** and store the tubes on ice.
11. Heat the three tubes containing the Laemmli-SDS sample buffer for 3- 5 mins in a PCR machine at 95°C and store the samples at -20°C until next week.

## Bradford Protein Assay

1. Place six 5 ml glass tubes in a rack and label 0, 20, 40, 60, 80, and 100  $\mu\text{g}$ . Add appropriate volumes of a 1 mg/ml BSA (bovine serum albumin) standard protein solution to each tube respectively. (One set-up for every two groups)
2. Add two more tubes to the rack and label *Drosophila* and 10% BCS/FBS. Place 10  $\mu\text{l}$  each of the *Drosophila* supernatant and 10% BCS/FBS from the left-over in steps 6 and 8 above into the two tubes separately.
3. Bring the volume in each glass tube to 100  $\mu\text{l}$  with distilled H<sub>2</sub>O.
4. Add 3 ml Bio-Rad protein assay dye and mix well.
5. Let stand for 15 min, read the OD<sub>595</sub> of the standard BSA samples and the *Drosophila* and BCS/FBS samples in the spectrophotometer.

## Homework:

1. Prepare a standard curve using the concentrations of the known samples (0, 20, 40, 60, 80, and 100  $\mu\text{g}$  = 0, 0.2, 0.4, 0.6, 0.8 and 1  $\mu\text{g}/\mu\text{l}$  (mg/ml)) (X-axis) and the OD<sub>595</sub> values (Y-axis).
2. Calculate the concentration ( $\mu\text{g}/\mu\text{l}$ ) and total protein content ( $\mu\text{g}$ ) of the three SDS-PAGE samples you have stored. (Note: You added the 3X laemmli sample buffer as an additional volume to the samples after you removed the aliquot!)

## Week 2: SDS-polyacrylamid gel electrophoresis (SDS-PAGE) and Western blot

### Objective

The objective of this lab exercise is to separate protein samples through SDS-PAGE and transfer the protein from gel into nitrocellulose membrane

### Protein SDS-Polyacrylamide Gel Electrophoresis

1. Prepare gel plates using 1.5 mm spacers.
2. Prepare and pour a separating gel.  
Add the following to a 125 ml sidearm flask:  
7.5 ml 1 M Tris pH 8.8  
10.0ml 30%:0.8%::acrylamide:bisacrylamide  
0.3 ml 10% SDS  
12.1ml H<sub>2</sub>O  
Degas the gel mixture and add:  
100.0µl 10% ammonium persulfate (AP)  
37.0µl 100% tetramethylethylenediamine (TEMED)  
Swirl gently and pour into gel sandwich.
3. Overlay (2 µl TEMED and 20 µl 10% AP in 4 ml H<sub>2</sub>O) and allow to polymerize one hour.
4. Pour off the overlay from the separating gel. Prepare and pour a stacking gel.
5. Add the following to a 50 ml sidearm flask:  
3.3 ml 1 M Tris pH 6.8  
2.0 ml 30%:0.8%::acrylamide:bisacrylamide  
0.2 ml 10% SDS  
14.3ml H<sub>2</sub>O  
Degas and add:  
100.0µl 10% AP  
80.0µl 100% TEMED
6. Swirl gently and pour into gel sandwich. Pour the gel solution to about 1/2 inch from the top of the plates and insert a 20-well comb to make sample wells.
7. Overlay with the same solution as in step 3 and allow polymerizing one-half hour.

### Transfer of proteins to membranes (Western blotting)

1. Place two pre-made 12-well gels (max 20 µl) in the apparatus.
2. Fill the apparatus with running buffer (0.025 M Tris-base, 0.192 glycine, 0.1% SDS) and wash wells with running buffer.
3. Load molecular weight markers, 20 µg *Drosophila*, 0.5 µg 0.1% BCS/FBS and 5 µg 1 % BCS/FBS samples.
4. Electrophorese the samples at 100 V for ~1 hr until the bromophenol blue tracking dye is at the bottom of the separating gel. Turn off the power and

remove the gel plates from the electrophoresis apparatus.

5. Transfer the gel to the blotting holder on top of a nylon batt and one layer of pre-wet blotting paper. Make sure there are no air bubbles between the gel and the blotting paper.
6. Carefully lay a piece of pre-wet transfer nitrocellulose membrane on top of the gel, being careful not to trap air bubbles between the membrane and the gel. Lay another piece of pre-wet blotting paper and the second nylon pad over the gel and close the holder. Remember which side the membrane is on.
7. Slide the holder into the slot in the blotting chamber. The chamber should contain about 1 liter of blotting buffer (0.025M Tris, 0.192 M glycine, 20% methanol, pH 8.3). Make sure that the membrane side of each holder is in the same orientation.
8. Place the frozen Bio-Ice in position.
9. Connect the lid and electrodes to the apparatus with the positive electrode on the same side as the membrane.
10. Blot at 100 V for 45 mins for nitrocellulose membrane.
11. Turn off the power and disassemble the holders carefully. **Mark the wells using a pencil.**
12. Transfer each membrane into a staining box and cover with Poinceau S stain (0.2% Poinceau S, 3% trichloroacetic acid (TCA), 3% sulfosalicylic acid). Allow it to stand for 10 minutes.
13. Pour excess stain back into the bottle. Rinse the membrane with distilled water until no more stain washes off. Proteins should be stained pink.
14. Keep the membrane in a plastic container (keep it moist) and store at 4 °C. **You should take a picture of the membrane to record the pattern of Poinceau S staining. (You should put a ruler next to your marker and always wear gloves when handling the membrane.)**

The molecular weight of proteins can be determined by preparing a molecular weight standard curve from the molecular weight standard markers electrophoresed on the gel along with the experimental samples. Either from the gel itself or from photographs of the gel a standard curve is prepared by measuring the **distance** migrated of each known protein (X-axis) and graphing that versus the **log<sub>10</sub>** of each protein's molecular weight (Y-axis).

#### Molecular Weight Standards - pre-stained recombinant proteins

- |                  |                  |
|------------------|------------------|
| 1. 250 Kd (blue) | 6. 37 Kd (blue)  |
| 2. 150 Kd (blue) | 7. 25 Kd (pink)  |
| 3. 100 Kd (blue) | 8. 20 Kd (blue)  |
| 4. 75 Kd (pink)  | 9. 15 Kd (blue)  |
| 5. 50 Kd (blue)  | 10. 10 Kd (blue) |

## **Week 3: Immunodetection of BSA protein**

### **Objective**

Using anti-BSA to detect the BSA protein or its homologs on the Western blot membrane.

### **Immunoassay of Western-blotted Proteins**

1. Place the membrane in a plastic box slightly larger than the membrane.
2. Add 15 ml of blocking buffer (10% bacto-peptone in 1X PBS, 0.1% Triton X-100) to the box. The membrane should be completely covered with the buffer. Place the box on the shaker for 50 min at RT.
3. Pour off the blocking buffer and wash the blot 3 x 5 min with 15 ml washing buffer (1X PBS, 0.1% Triton-X 100) with shaking at RT.
4. Pour off the washing buffer and add 15 ml 1:500 final dilution of the 1<sup>st</sup> antibody (rabbit anti-bovine serum albumin Ab) diluted in antibody buffer (1% bacto-peptone in 1X PBS, 0.1% Triton X-100). Incubate for 50 min with shaking at RT.
5. Pour off the 1<sup>st</sup> antibody solution and wash 3 x 5 min with 15 ml washing buffer at RT.
6. Add 15 ml of the 2<sup>nd</sup> antibody (alkaline phosphatase conjugated goat anti-rabbit IgG Ab) diluted 1:20,000 in antibody buffer. Incubate for 50 min with shaking at RT.
7. Pour off the 2<sup>nd</sup> antibody solution and wash 3x 5 min with washing buffer.
8. Wash 2 x 1 min with 15 ml predetection buffer.
9. Add 15 ml detection buffer and place the blot in the dark. Store the membrane wrapped in Saran wrap. Rewetting the membrane in water can enhance the blue.

### 1X PBS Predetection Buffer

0.13 M NaCl            100 mM Tris pH 9.5  
7 mM Na<sub>2</sub>HPO<sub>4</sub> 100mM NaCl  
3 mM NaH<sub>2</sub>PO<sub>4</sub> 10mM MgCl<sub>2</sub>

### Detection Buffer

- 25 µl 75 mg/ml nitro blue tetrazolium in 70% dimethylformamide and 25 µl 50 mg/ml 5-bromo-4-chloro-1-indolyl phosphate in 100% dimethylformamide per 25 ml of the predetection buffer
- 200 µl/ 10 ml predetection buffer from the commercial available premade substrate for AP.

**Questions that should be answered in the lab report:**

1. How many proteins can be detected in the *Drosophila* and BCS/FBS samples using Poinceau S stain?
2. How many proteins in the BCS/FBS sample appear to be recognized by the anti-BSA Ab? Explain this result.
3. How many proteins in the *Drosophila* sample appear to be recognized by the anti-BSA Ab? Explain this result.
4. Using the molecular weight standards, prepare a  $\log_{10}$  mol wt (Y-axis) vs. distance migrated or  $R_f$  value (X-axis) standard curve.
5. What is the distance migrated of the main reacting antigen in the BCS/FBS sample? What is that protein's molecular weight? Are there other bands? Explain.
6. Does the information in question 5 strengthen or weaken your explanation for question 1? Explain.
7. To what level of confidence could you estimate that a sample does not contain any protein that will cross-react with the antibody used?