LAB MODULE III. PROTEOME ANALYSIS IN ZEBRAFISH

OBJECTIVES: After completing this module, you should be able to
1. Properly make a buffer suitable for tissue homogenization.
2. Homogenate tissues for basic cell fractionation.
3. Differentiate between a cell homogenate (cell extract), supernatant and pellet.
4. Describe the role of centrifugation in a cell biology lab.
5. Identify and differentiate between swinging bucket and fixed angled rotors.
6. Identify tubes appropriate for use in a rotor vs. those that should not be used in rotor.
7. Identify and describe the use of all laboratory equipment and solutions used in the isolation of cellular proteins.
8. Determine protein concentration of cell fractions by constructing a standard curve.
9. Properly set up, run and interpret an SDS-PAGE of cell fractions.
10. Interpret the results of a Western blot.
11. Read primary literature and present information in a PowerPoint presentation.

REFERENCES:
Basic Laboratory Methods for Biotechnology
ECB: Panel 4-3 Cell breakage and initial fractionation of cell extracts (pgs. 160-161)
ECB: Panel 4-4 Protein separation by chromatography (pgs. 162-163)

EXERCISES:
1. Reading and reporting on a primary journal article.
2. Making a buffer
3. Prepare cell fractionation samples
4. Determine the protein concentration using a Lowry proteins assay
5. Run a SDS-PAGE
6. Western Blotting

PROTEOMES (A Brief Introduction)
In this module entitled “Proteome Analysis in Zebrafish”, we will be investigating the response of cells to stress at the molecular and cellular levels. Cells respond to stress such as elevated temperatures by expressing a family of proteins referred to as heat shock proteins. Many of the proteins induced during heat shock are characterized as chaperones that facilitate the proper folding of proteins during conditions of cell stress, including heat shock, but also during other types of stress such as exposure to environmental toxins. As part of this module you will be do a number of activities as shown outlined in the list of exercises presented above.

The first exercise includes looking up and reading at least three journal articles from the primary research literature that is representative of how cells respond to a particular type of stress. In addition you will be responsible for familiarizing yourself with the individual methods and techniques presented in these papers that represent how responses can be investigated in the molecular cell laboratory. At the end of this module each student will be required to present a PowerPoint presentation summarizing the results of at least one of the journal articles in a public presentation.
In exercises two you will be responsible for performing several basic skills related to using the analytical balance and pH meter in order to make a biological buffer. This buffer will be used in the homogenization of various tissue samples taken from organisms that have been exposed to a moderately elevated temperature as well as control organisms that have not been exposed to elevated temperature.

Exercise three will focus on preparing cell fractions from homogenated tissues isolated from both control and heat treated organisms. This exercise will include the proper use of a preparative centrifuge and the proper handling of biological samples in order to avoid protein degradation.

Exercise four includes determining the protein concentration of each of the samples prepared above in order to further characterize the proteins in each sample by SDS-PAGE and Western blot analysis. During this exercise a review of the proper use of a spectrophotometer and the preparation of a standard curve will be performed.

The last two exercises of the module will include the characterization and analysis of the protein expression profiles from both control and treated organisms. Part of the last two exercises will be performed by the instructor and the results discussed in class.

EXERCISE DETAILS:
Please refer to the presentation provided during lab for instructions on completing exercise one. Anyone missing this laboratory presentation will be responsible for getting the information from someone else. Forms required for the completion of this exercise can be found at Drive O:\Biology\Daggett\BIO 215 Spring 2008. Completed journal article forms for each of the three required articles will be turned in for points that will be recorded as part of the lecture written assignment component of your grade. None of these forms will be dropped and no forms will be accepted after your PowerPoint presentation.

Instructions for other exercises will be provide during laboratory demonstrations. Each student is responsible for recording the steps involved in their laboratory books. Failure to record these instructions in your lab book may result in lost points.

1. READING PRIMARY JOURNAL ARTICLES

2. BUFFER PREPARATION
Outline in your lab books the step by step process for how you would go about making 50 mls of the following buffer. Be prepared to make this or a similar buffer in a group or as an individual.

Note that you have the following reagents available.
- a bottle (dry powder) of PIPES (MW 302.37)
- a bottle (liquid) of 1M KOH
- a bottle (dry powder) of Magnesium Sulfate (FW 246.6)
- a bottle (liquid) of 0.5M EDTA

50 mM PIPES/K+ pH 7.3
1 mM MgSO4
4 mM EDTA
3. **SAMPLE PREPARATION** (This procedure is subject to change depending on the availability of materials.)

1. Prepare PIPES/K+ pH 7.3 buffer (50 mM PIPES/K+ pH 7.3, 1 mM MgSO4, 4 mM EDTA)
2. Prepare anesthetic: 100 mg (ethyl 3-aminobenzoate methanesulfonic acid) / 100 mls aquarium safe water.
3. Immerse fish in anesthetic until unresponsive.
4. Dissect out selected tissues. KEEP TISSUE ON ICE.
5. Determine the amount of buffer required to add 4X the tissue volume. (Record the weight of the tissue sample in grams: __________

   Multiply the above value by 4, add this volume in mls: ______________

   Example: .4 g tissue x 4 = 1.6 mls buffer

6. Prepare working buffer by adding protease inhibitors (Currently a 1000X stock).

   Example: For 2.0 mls of buffer add 2 uls of the 1000X protease inhibitors

7. Add the working buffer to each tissue sample.
9. Place homogenate in a pre-chilled 15 ml. centrifuge tube and centrifuge at 200 x g for 5 minutes at 4° C to pellet the unbroken cells and tissue fragments.
10. Decant the supernatant into a clean centrifuge tube. Take a 200 ul sample of the 200 x g super. Throw the pellet away.
11. Centrifuge the 200 x g supernatant at 1000 x g for 7 minutes. Decant the supernatant into a new tube. Take a 200 ul sample of the 1000 x g supernatant.

You should have the following samples from both control (C) and heat treated (T) organisms.

<table>
<thead>
<tr>
<th>Control samples (C)</th>
<th>treated samples (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1: 200 x g super</td>
<td>T1: 200 x g super</td>
</tr>
<tr>
<td>C2: 1000 x g super</td>
<td>T2: 1000 x g super</td>
</tr>
</tbody>
</table>
4. PROTEIN ASSAY

Objective: To prepare samples for SDS-PAGE and Western Blot analysis

* Keep all samples on ice at all times.

**Protein Assay Procedure:**

1. Set up test tubes according to the following table

<table>
<thead>
<tr>
<th>Tube #</th>
<th>BSA (1.0 ug/ul)</th>
<th>dH2O (ul)</th>
<th>Conc. %T</th>
<th>Ab750 (A = -log t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>95</td>
<td>5 ug</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>90</td>
<td>10 ug</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>80</td>
<td>20 ug</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>70</td>
<td>30 ug</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>60</td>
<td>40 ug</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>50</td>
<td>50 ug</td>
<td></td>
</tr>
</tbody>
</table>

**Samples**

- C1 2 uls 98
- C2
- T1
- T2

2. Prepare fresh Lowry reagent by mixing, in the following order:
   - (A) 2% (w/v) Na2CO3 in 0.1 N NaOH 100.0 ml (make fresh each time)
   - (B) 1 % (w/v) CuSO4 · 5 H2O 1.0 ml
   - (C) 2% (w/v) Sodium tartrate 1.0 ml

3. Add 2.5 ml Lowry reagent to each tube. Mix well and incubate 10 minutes at room temperature.

4. Add 0.25 ml (250 ul) of FCP (make fresh) and rapidly mix. Allow to sit at room temperature for 30 minutes in the dark (put in a drawer).

5. Read absorbency at 750 nm. (You must use a spec with a red filter and the correct lamp)

6. Graph your results using the Protein Assay Template and determine the protein concentrations for each of your samples. (Template is available at Drive O:\Biography\Daggett\BIO 215 Spring 2008)

**NOTES**

The Lowry method depends on the presence of tyrosine within the protein to be measured. The standard protein must contain approximately the same number of tyrosine residues as the sample, or the procedure will be inaccurate. If there are no tyrosine residues in the sample to be measured, the Lowry method of protein determination is useless, and use should be made of the Bradford assay. In general, the Bradford assay is the method of choice for protein determinations.
**MATERIALS DETAILS FOR LOWRY**

Samples to be assayed
- 2% (w/v) Na₂CO₃ in 0.1 N NaOH (0.4 g NaOH in 100 ml dH₂O + 2 g Na₂CO₃)
- 1% (w/v) CuSO₄·5 H₂O (1 g/100 mls dH₂O)
- 2% (w/v) Sodium tartrate (2 g/100 mls dH₂O)
- Folin-Ciocalteu phenol reagent diluted 1:1 with dH₂O (FCP)
- Bovine Serum Albumin (BSA) (1 mg/ml) or Immunoglobulin (1 mg/ml)
- Spectrophotometer and tubes
- Micropipettes and tips

**Prepare samples for SDS-PAGE:** Each well in your gel will be loaded with the same amount of protein (30 ug/lane). Determine the protein concentration of your samples by performing a protein assay. A standard curve must be included every time the protein concentration of a sample is to be determined.

![Lowry Assay Standard Curve](image)

Using the equation derived from your standard curve, calculate the concentration of your protein samples. The graph shows a standard curve in which the $A_{750}$ vs. protein concentration was plotted. Using the equation of this line ($y = 0.0053x$) the protein concentrations of the samples may be calculated. The $A_{750}$ for each of your samples = $y$ and you solve for $x$. The value for $x$ you calculate will be the concentration of protein in 2 uls of your sample, therefore divide by 2. This will give you ug/1 l (mg/ml).

Write down the equation of the line from your standard curve: ______________

Calculate the protein concentration (ug/ul) for

sample C1: (200xg supernatant) __________
sample C2: (1000xg supernatant) __________
sample T1: (200xg supernatant) __________
sample T2: (1000xg supernatant) __________
GATHER REAGENTS & MATERIALS

4X Tris.Cl/SDS, pH 8.8
4X Tris.Cl/SDS, pH 6.8
30% acrylamide/0.8% bisacrylamide (30:1)
Ammonium persulfate, 10% (0.1 g/1 ml dH2O, Make fresh each time.)
Temed
SDS electrophoresis buffer, 1X (5X stock available)
BioRad Mini-PROTEAN 3 Cell
pipettors w/tips (P1000, P100, and P10)
aspirator

Reference: Bio-Rad Handouts
GEL CASSETTE PREPARATION

Read and follow manufacturer's instructions.

- Sections 1. General Information (1.1 Introduction, 1.2 Components, 1.3 Specifications, 1.4 Chemical Compatibility, 1.5 Safety)
- Section 2. Set Up and basic Operation (2.1 Gel Cassette Sandwich Preparation Hand Cast Gels Discontinuous Polyacrylamide Gels)
- Section 2.2 Mini-PROTEIN 3 Electrophoresis Module Assembly and Sample Loading
- Section 2.3 Gel Electrophoresis
- Section 3. Separation Theory and Optimization (3.1 and 3.2)
- Section 4. Follow instructions given in this handout not in the Bio-Rad handout
- Sections 5, 6, 7 read.

Replace Section 4 with the following information:

SEPARATING GEL (variable %)
1. In a 50 ml conical tube, add 2.5 mls 4X Tris.Cl/SDS, pH 8.8.
2. Add the 30% acrylamide/0.8% bisacrylamide according to the final % gel
   - 8% gel = (8%)(10 mls) = (x)(30%), x = 2.67 mls
   - 10% gel = (10%)(10 mls) = (x)(30%), x = 3.33 mls
   - 12% gel = (12%)(10 mls) = (x)(30%), x = 4.0 mls
3. Bring to 10 mls with dH2O.
4. Degas under vacuum 15 minutes.
5. Immediately prior to pouring gel, add 50 uls 10% APS and 5 uls TEMED. Invert tube gently to mix.
6. Apply acrylamide mix to gel cassette using a transfer pipette.
7. Carefully overlay each gel with 200 uls dH2O using a gel loading tip.
8. Polymerize for 45 minutes.
9. Rinse off surface of gel and wick dry.
10. Pour stacking gel.

STACKING GEL (4.0% acrylamide)
1. In a 15 ml conical tube, add 1.25 mls 4X Tris.Cl/SDS, pH 6.8
2. Add 666 uls mls 30% acrylamide/0.8% bisacrylamide
3. Bring to 5 mls with dH2O.
4. Wash the surface of the separating gels with dH2O. Remove excess water.
5. Add 25 uls 10% APS and 5 uls TEMED to gel solution. Swirl gently to mix.
6. Overlay each gel with stacking gel solution.
7. Insert combs and allow to polymerize for 60 minutes.
Recipes:

**4X Tris Cl/SDS, pH 8.8**
Dissolve 45.5 g Tris base (1.5 M) in 150 mls H2O. Adjust pH to 8.8 with 1N HCl. Add H2O to 250 ml total volume. Filter solution through a 0.45 um filter, add 1 g SDS [0.4% (w/v)], store at 4°C up to 1 month.

**4X Tris Cl/SDS, pH 6.8**
Dissolve 6.05 g Tris base (0.5 M) in 40 mls H2O. Adjust pH to 6.8 with 1N HCl. Add H2O to 100 ml total volume. Filter solution through a 0.45 um filter, add 0.4 g SDS [0.4% (w/v)], store at 4°C up to 1 month.

**Ammonium persulfate, 10%**
100 mg ammonium persulfate in 1 ml H2O. Make fresh each time.

**SDS electrophoresis buffer, 5 X**
15.1 g Tris base (0.125 M)
72.0 g glycine (0.96 M)
5.0 g SDS [0.5 (w/v)]
H2O to 1000 ml
Dilute to 1X before use.
Do not adjust the pH of the stock solution, as the pH is 8.3 when diluted. Store at 0° to 4°C until use.

**SDS sample buffer, 6X**
7 ml 4X Tris.Cl/SDS, pH 6.8
3.0 ml glycerol [30% (v/v)]
1 g SDS [1% (w/v)]
0.93 g DTT (0.5 M)
1.2 mg bromphenol blue [0.0012% (w/v)]
Add H2O to 10 ml if needed
Store in 0.5 ml aliquots at -70°C.

**Coomassie Blue Stain**
Methanol 50% (v/v) 500 mls
Coomassie brilliant blue R-250 0.05% 0.5 g
Acetic acid 40% (v/v) 400 mls
dH2O 100 mls

**Destain**
Methanol 250 ml
Acetic acid 100 ml
dH2O 650 ml
### Solutions for Western Blotting

<table>
<thead>
<tr>
<th><strong>Transfer buffer (PVDF membrane)</strong></th>
<th><strong>Nitrocellulose membrane</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>18.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>86.5</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4,000 ml</td>
</tr>
<tr>
<td>15% MeOH</td>
<td>900 mls</td>
</tr>
<tr>
<td>dH₂O</td>
<td>qs 6,000 mls</td>
</tr>
<tr>
<td>10% MeOH</td>
<td>(600 mls)</td>
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</tbody>
</table>

**Tris-Buffered Saline (TBS) pH 7.4**

- 100 mM TrisCl pH 7.5 (.1M)(121.1)(4) = 48.4 g
- 0.9% NaCl (0.9 g/100 mls) = 40.0 g
- dH₂O = 4 liters

**TTBS (0.5% Tween -20)**

Add .5 mls Tween-20/ 100 mls or 5 mls/liter

**Blocking Buffer**

TTBS w/ 10% carnation milk (10 g/100 mls TTBS)
SAMPLE PREPARATION
1. Determine the protein concentration.
2. Add 6X sample buffer (1 µl of 6X SB/5 µls sample, 1/6 or 1:5)
3. Determine final protein concentration of sample = Original concentration x 0.833.
4. Calculate the volume of sample to add to each well. (30-50 µg/ well)
For some applications, for example zebrafish embryos, lanes may be loaded on a per embryo basis instead of a particular protein concentration.
5. Denature protein (Bring water to boil, turn off heat, place samples in water for 5-10 minutes.)
6. Run the gel at 200 volts until the dye front reaches the end of the gel (~ 40 minutes)
7. Remove gel, cut off stacking gel, and place separating gel in CB stain overnight, then destain.
8. Photograph gel.

Record the following information in your lab books
GEL #: ____________.
DATE: ____________.
% ACRYLAMIDE ____________.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Extract ID</th>
<th>Protein conc.</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
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<td></td>
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<tr>
<td>10</td>
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</tr>
</tbody>
</table>
WESTERN BLOTTING

1. Cut PVDF membrane to cover gel.
2. Wet membrane in 100% MeOH for 30 seconds, rinse in dH₂O for 3 minutes, then soak in transfer buffer for 5 minutes.
3. Assemble transfer apparatus as follows (all materials must be saturated with transfer buffer):
   - Black grid down
   - Scotch bright pad
   - Filter paper
   - Gel
   - Membrane
   - Filter paper
   - Scotch bright pad
   - White grid on top
4. Insert so that white grid faces the red electrode, run for 1 hour at 100 Volts.
5. Remove membrane, fix in 100% MeOH for 1 minute (Membrane can be air-dried and stored at 4°C if needed).
6. Wash fixed membrane in small amount of blocking buffer for several minutes, then incubate in fresh blocking buffer for at least one hour.
7. Add primary antibody diluted at the recommended ratio in Blocking buffer.
8. Incubate overnight at 4°C with shaking (Place a stir-plate in the refrigerator in room 208).
9. Remove primary antibody, wash with TTBS 3 X 15’
10. Add secondary antibody diluted at the recommended ratio in TTBS.
11. Incubate for at least 1 hour at room temperature.
12. Remove secondary antibody, wash with TTBS 3 X 15’