**ENY6822C**

**Molecular Techniques of Invertebrates and their Pathogens:**

**Western blotting**

**Summer A 2016**

**Leigh Boardman and Hahn lab protocols**

**Protein extraction: Extract total proteins from *Drosophila* samples**

*Work on ice!*

*If samples will be stored, add proteinase inhibitors to the PBS.*

1. For each sample, homogenize 10 larvae in 150 μl of PBS buffer. Use bead beater.

2. Centrifuge the homogenate at 13000rpm for 30min at 4°C

3. Transfer supernatant to a new tube – try to leave the fat behind

4. Discard beads and bug mush. Store supernatant overnight at -80°C

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**Protein quantification: Measure protein concentration of supernatant using bicinchoninic acid (BCA) assay**

*See: Pierce BCA Protein Assay Kit.pdf – available from thermofisher.com*

1. Prepare the Diluted Albumin (BSA) Standards

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Dilution scheme for Microplate Procedure (Working Range = 20-2000 µg/ml)* | | | | |
| **Vial** | **Volume of diluent (µl)**  **dH2O** | **Volume (µl) and source of BSA** | | **Final BSA concentration (mg/ml)** |
| A | 0 | 150 | Stock | 2 |
| B | 62.5 | 187.5 | Stock | 1.5 |
| C | 162.5 | 162.5 | Stock | 1 |
| D | 87.5 | 87.5 | Vial B dilution | 0.75 |
| E | 162.5 | 162.5 | Vial C dilution | 0.5 |
| F | 162.5 | 162.5 | Vial E dilution | 0.25 |
| G | 162.5 | 162.5 | Vial F dilution | 0.125 |
| H | 200 | 50 | Vial G dilution | 0.025 |
| I | 200 | 0 |  | 0 = Blank |

2. Prepare three 100 µl dilutions of each of your protein supernatant samples: 1X, 10X, 20X.

*Remember to mix your sample before pipetting*

*100 µl is enough for 3 technical replicates on the plate*

3. Prepare the BCA Working Reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).

*Calculate how much WR you need: 200 μl of WR reagent is required for each sample in the microplate procedure*

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

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4. Pipette 25 μl of each standard or unknown sample replicate into a microplate well. Remember technical replicates.

*Remember to mix your sample before pipetting. Keep track of what you put in each well!*

5. Add 200 μl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds

6. Cover plate and incubate at 37°C for 30 minutes

7. Measure the absorbance at or near 562 nm on a plate reader

8. Calculate the protein concentration in your samples using a standard curve

*Helpful hints: Remember the dilution factor!*

*Use the TREND function in Excel OR use polynomial trendline to fit data*

9. Calculate how much supernatant (in µL) is needed to load 40µg protein into a well

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**Protein denaturation: denature the protein samples for SDS-PAGE**

1. Dilute \_\_\_\_ μL sample in an equal amount of 2X Laemmli buffer (with β-mercaptoethanol)

*This is determined in step 9 above. This must be less than 15µL as the well can hold a maximum of 30µL.*

2. Heat at 95°C for 5 min

3. Store at -80°C if needed

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**SDS-PAGE (Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis): Separates the proteins based on molecular weight**

1. Defrost samples

2. Reboil samples at 95°C for 2 min

3. Setup precast gel in system

4. Fill tank with Tris/Glycine/SDS running buffer

**1X Tris/Glycine/SDS running buffer:** 100ml 10X buffer + 900ml dH2O

5. Load sample and protein weight marker into gel

*Keep track of sample order in wells!*

6. Place the lid on the tank and connect the leads to the powerpack. Run the gel at 75V for 5 min

7. Increase the voltage to 150V and run the gel until the front has run off the bottom (~1h)

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**Western transfer: tank blotting protein gels to PVDF membranes**

1. Make sure bio-ice cooling unit is filled with water and frozen at -20°C.

2. After gel has completed running, switch off apparatus. Disconnect power!

3. Wet the PVDF membrane by placing it in methanol for 15-30 seconds.

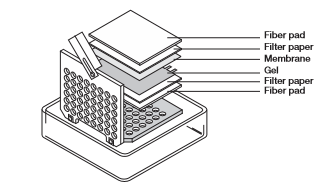
*Make sure not to touch the membrane. Wear gloves and only handle the membrane at the very edges with hands or forceps. Do not allow the membrane to dry off!*

4. Soak the gel, membrane, filter paper, and fiber pads in transfer buffer for 1 hour.

**1X Tris/Glycine transfer buffer**: 100ml 10X Tris/Glycine buffer + 700 ml dH2O

Make this ahead of time and cool it to 4oC before use.

5. Prepare the cassette, the hinge will be upright when fitted into the electrode apparatus. Place 1 pad then 1 piece of filter paper then the gel, then the membrane, then another piece of filter paper, and the last pad. Close the sandwich and place into electrode apparatus with the membrane closest to the red side so the protein transfers from the gel to the membrane. Be careful at each step to eliminate air bubbles between any of the layers, especially between the gel and membrane. To eliminate air bubbles, you can add a bit more transfer buffer and roll them out with a pasteur pipette rolling-pin style. Do not allow anything to dry off during this process.



**Very important: Membrane must be closest to the CLEAR side of the cassette**

**Clear side of cassette closest to RED side of setup**

6. Add the ice pack to the buffer tank and fill with transfer buffer.

7. Place a stir bar in the buffer tank and get it mixing well on a stirring plate.

8. Run at 100V for 60min

9. After transfer, carefully disassemble setup. Cut the top left corner of the membrane to give you orientation. This is essential as the proteins will only be bound to one side of the membrane.

10. Wash the membrane by placing it in ultra-pure water and shaking it for 5 minutes. Repeat the washing step three times. Move straight to immunodetection, or dry the membrane at room temp for about an hour then store at -20oC until detection. Membrane will be good in the freezer for several weeks, but must be rewet in methanol before use.

11. Stain the gel overnight to visualize remaining protein

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**Antigen detection: Western blotting detection of antigens blotted to PVDF membranes**

*Make sure not to touch the membrane. Wear gloves and only handle the membrane at the very edges with hands or forceps. Do not allow the membrane to dry off!*

*Make all working solutions fresh on the day of use*

1. Cut the membrane between the protein of interest (ATG8, 12/14kDa) and loading control (tubulin, 55 kDa). Very important to cut the top left corner of the bottom strip of membrane too.

*The top part of the membrane will be used to detect tubulin, and the bottom (smaller) part ATG8*

2. Wetting- wet the dry membrane in approximately 30ml 50% methanol.

3. Washing- wash the blot for 10 min in approximately 30ml **1xTBS** for 10 minutes shaking at room temperature, pour off solution and repeat.

Make 500ml of **1xTBS** by placing 50ml 10x in 450ml ultrapure water.

4. Blocking- Place membrane in 20ml **blocking solution** and shake at room temperature for 1 hour then decant solution.

Make 50ml **blocking solution** by adding 0.1g dry milk to 50ml TBS.

5. Washing- Wash in 30ml of Tween-TBS (**TTBS**) for 10 minutes with agitation at room temperature, pour off solution.

Make 240 ml of Tween-TBS (**TTBS**) add 0.24ml Tween-20 to 240ml TBS and mix well.

6. Primary antibody incubation- add 20ml **antibody buffer** to the membrane and then add primary antibody. Incubate for 2 hours with gentle agitation, pour off.

• Anti-ATG8: Add 20 µl for a 1:1000 dilution

• Anti-tubulin: Add 2 µl for a 1:10000 dilution

Make 50ml **antibody buffer** by adding 0.1g non-fat dry milk to 50ml of TTBS.

7. Washing- Wash in 30ml of **TTBS** for 10 minutes under agitation at room temp, then repeat.

8. Secondary antibody incubation- add 20ml **antibody buffer** to the membrane and then add secondary antibody. Incubate while shaking at room temperature for 1 hour, then pour off.

• Anti-ATG8: 2 µl of secondary goat-anti-*rabbit* antibody for a 1:10000 dilution

• Anti-tubulin: 2 µl of secondary goat-anti-*mouse* antibody for a 1:10000 dilution

Make 50ml **antibody buffer** by adding 0.1g non-fat dry milk to 50ml of TTBS.

9. Washing- Wash in 30ml of **TTBS** for 10 minutes under agitation at room temp, then repeat two more times (three washes total).

10. Add equal parts (~2.5 mL each) of each bottle from the ECL substrate reagents to a tube

11. Remove membrane and let excess liquid drip off, but do not let it dry. Place in large petri dish (or similar) and incubate with ECL detection solution for 1 min at room temperature.

12. Remove membrane and let excess liquid drip off. Place in plastic sleeve. Squeeze out excess liquid and seal the bag making sure to get out all the wrinkles and air bubbles. Wash the outside of the bag, dry it off and expose in imaging system.