**Protein Extraction and Calibration Curve**

*If storing samples for more than 30 days, add a protease inhibitor to PBS.*

* Turn on low temperature centrifuge. Weigh and label new vials.
* In 300μL of PBS homogenize 1 larva using a bead beater for 30secs
* Centrifuge the homogenate at 13000rpm for 30min at 4°C
* Transfer supernatant to a new tube, leave behind fat, and discard beads and bug mush.
* **Store supernatant overnight at -80°C**

**Protein Quantification using BCA kit** *See: Pierce BCA Protein Assay Kit.pdf*

Turn on incubator to 37°C and prepare the BSA solution standards and unknowns

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 | 75 | 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 |

* Prepare three 100µl dilutions of each of your protein supernatant samples: 1X, 10X, 20X.
  + [diluting volume/#’x’ volume] = diluted volume
  + Ex: 100µl/1x = 100µl of unknown need to be added
  + Ex: 100µl/10x = 10µl of unknown need to be added
  + Ex: 100µl/20x = 5µl of unknown need to be added
* Prepare the BCA Working Reagent (WR) 50:1, Reagent A:B. 200µl needed per sample plus 30%
  + Ex: 0.02\*A=vol of B
  + (#stds + #unk)\*(3rep)\*(vol of WR/sample) = vol WR needed + 30%
* Mix each sample for 5secs and pipette 25μl of each standard or unknown sample in triplicate onto microplate well.
* Add 200 μl of the WR to each well, place on a plate shaker for 30 seconds at 2000rpm
* Cover plate with *Press’n Seal* and incubate at 37°C for 30 minutes
* Turn on hot plate
* Measure the absorbance at or near 562 nm on a plate reader
  + Add wavelength to datasheet before saving to drive
* Calculate the protein concentration in your samples using a standard curve and determine ‘x’μL needed provide 40μg per well

|  |  |  |  |
| --- | --- | --- | --- |
| Dilution factor = | Volf  = | Vol of stock + dilution = | 100μL stock + H2O |
|  | Voli | Vol of stock | X μL stock |

* + (Dilution factor) \* (Concentration) = μg of stock
  + Things to remember:
    - ug/ul = mg/ml
    - plot OD on y-axis, concentration on x-axis
    - make scatter plot>marked>select date>add>select x, select y
* Dilute ’x’ μL sample in an equal amount of 2X Laemmli buffer (with β-mercaptoethanol)

*Must be less than 15µL as the well can hold a maximum of 30µL.*

* Heat at 95°C for 5 min
* **Store at -80°C if needed**
  + *if storing for more than 30 days inhibitors need to have been added to PBS*

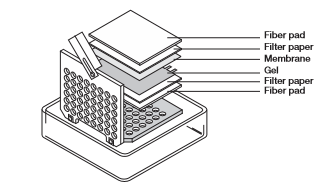
**SDS-PAGE (Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis)**

* Turn on heating block to 95°C
* Defrost samples
* Reboil samples at 95°C for 2 min
* Setup precast gel in system
* Fill tank with 1X Tris/Glycine/SDS running buffer
  + 100ml 10X buffer + 900ml dH2O
  + C1V1=C2V2
* Load sample and protein weight marker into gel
  + Place the lid on the tank and connect the leads to the powerpack. Run the gel at 75V for 5 min
  + Increase the voltage to 150V and run the gel until the front has run off the bottom (~1h)

**Transfer proteins from SDS gel to PVDF membranes**

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

* Make transfer buffer and cool to 4oC
  + **1X Tris/Glycine transfer buffer** = 100ml 10X Tris/Glycine buffer + 700 ml dH2O
* After gel has completed running, switch off apparatus. Disconnect power!
* Wet the PVDF membrane in MeOH for 15-30 seconds.
  + Wear gloves, handle membrane at edges, KEEP MEMBRANE WET
* Soak the gel, membrane, filter paper, and fiber pads in transfer buffer for 1 hour.
* 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**

* Add the ice pack and stir bar to the buffer tank and fill with transfer buffer and get it mixing well on a stirring plate.
* Run at 100V for 60min
* Cut the top left corner of the membrane for orientation.
* Wash the membrane by placing it in ultra-pure water and shaking it for 5 minutes. Repeat the washing step three times. 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.
* Or, stain the gel overnight to visualize remaining protein

**Detection of antigens blotted to PVDF membranes**

Wear gloves, handle membrane at edges, KEEP MEMBRANE WET

Make all working solutions fresh on the day of use

* 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*
* Make 50% MeOH
* Wash
  + Wetting- wet the dry membrane in approximately 30ml 50% methanol.
* Make 500ml of **1xTBS** by placing 50ml 10x in 450ml ultrapure water.
* Shake
  + Washing- wash the blot for 10 min in approximately 30ml **1xTBS** for 10 minutes shaking at room temperature, pour off solution and repeat.
* Make 50ml **blocking solution** by adding 0.1g dry milk to 50ml TBS.
* Block
  + Place membrane in 20ml **blocking solution** and shake at room temperature for 1 hour then decant solution.

**1° Antibody Incubation**

* Make 240 ml of Tween-TBS (**TTBS**) add 0.24ml Tween-20 to 240ml TBS and mix well
* Wash
  + Wash in 30ml of Tween-TBS (**TTBS**) for 10 minutes with agitation at room temperature, pour off solution.
* Make 50ml **antibody buffer** by adding 0.1g non-fat dry milk to 50ml of **TTBS**.
* Incubate
  + 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 (1° Ab:buffer)
  + Anti-tubulin: Add 2 µl for a 1:10000 dilution (1° Ab:buffer)
* Wash in 30ml of **TTBS** for 10 minutes under agitation at room temp, then repeat.

**2° Antibody incubation**

* Make 50ml **antibody buffer** by adding 0.1g non-fat dry milk to 50ml of **TTBS**
* 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-X-*rabbit* ab for a 1:10000 dilution
  + Anti-tubulin: 2 µl of secondary goat-X-*mouse* ab for a 1:10000 dilution
* Wash
  + Wash in 30ml of **TTBS** for 10 minutes under agitation at room temp, then repeat two more times (three washes total).
* Make ECL by adding equal parts (~2.5 mL each) of each bottle from the ECL substrate reagents to a tube
* ECL Incubation
  + 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.
* Visualize
  + 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.