**Protein Extraction and Calibration Curve**

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

* Gather the following before beginning;
  + Ice
  + 1x PBS
  + Inhibitors
  + Microtubes w/ caps
  + Parafilm
  + Petri dishes
  + Pipette and tips
* Stretch Parafilm across one petri dish
  + Set aside
* Add 500ul of PBS and 5ul of each inhibitor to an empty microtube
  + Hold tubes on ice
* With the larva constrained and folded in half, exposing the dorsum, make an incision through the cuticle at the proleg
* Force the lymph fluid through the incision onto the stretched Parafilm
* Pipette the hemolymph into the prepared microtubes
* Place the microtube on ice and repeat this process for all of the samples.
* Centrifuge at 4C, 15000rpm, 5mins
* Decant supernatant (careful to leave behind fat and precipitate) into clean labeled microtubes.
* **Store supernatant at -20°C**

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

Prepare the Diluted Albumin (BSA) Standards

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Dilution Scheme for Standard Test Tube and Microplate Protocols (Working Range = 100-1500μg/mL) | | | | |
| **Vial** | **Volume of diluent (µl)**  **dH2O** | **Volume (µl) and source of BSA** | | **Final BSA concentration (mg/ml)** |
| A | 0 | 300 | Stock | 2 |
| B | 125 | 375 | Stock | 1.5 |
| C | 325 | 325 | Stock | 1 |
| D | 175 | 175 | Vial B dilution | 0.75 |
| E | 325 | 325 | Vial C dilution | 0.5 |
| F | 325 | 325 | Vial E dilution | 0.25 |
| G | 325 | 325 | Vial F dilution | 0.125 |
| H | 400 | 100 | Vial G dilution | 0.025 |
| I | 400 | 0 |  | 0 = Blank |

* Standard Microplate Protocol (Working Range = 100-1500μg/mL)
  + 3 Reps of each dilution
  + 7 treatments plus 9 standards fit a 96 well plate

**\*\*\*WORK ON ICE TO REDUCE OXIDATION\*\*\***

* Prepare a consumable volume of Commassie reagent, allow it to equilibrate to room temperature (WR). 200µl needed per sample plus 30%
  + (#stds + #unk)\*(3rep)\*(250) +(0.3\*WR needed) = WR needed
* Prepare two 100µl dilutions of each of your protein samples in water: 1:2 and 1:10
  + 1:2 = 1 part sample for every 2 parts solution = 50 parts sample 50 parts water
  + 1:10 = 1 part sample for every 10 parts of solution = 10 parts sample 90 parts water
* Vortex each standard or unknown for 5 secs then pipette 5μL of each standard or unknown sample into the appropriate well of a microplate.
* Add 200μL of the Coomassie Reagent to each well.
  + Use multi-channel pipette to speed up the process
* Cover plate with *Press’n Seal,* mix with plate shaker for 30 seconds, then incubate at room temperature for 10 minutes.
* Measure the absorbance at or near **595** nm on a plate reader.
  + Add wavelength to datasheet before saving to drive.
* Remember to compensate for working reagent dilution:

|  |  |  |  |
| --- | --- | --- | --- |
| Dilution factor = | Volf  = | Vol of sample + dilution = | 5μL sample + 200μL Coomassie |
|  | Voli | Vol of sample | 5μL sample |

* + (Dilution factor) \* (Concentration) = μg of protein in sample
  + Things to remember:
    - µg/µl = mg/ml
    - plot OD on y-axis, concentration on x-axis
    - make scatter plot>marked>select date>add>select x, select y
* Calculate the protein concentration in your samples using a standard curve and determine μL needed provide 40μg per gel well.
  + [40/sample concentration]
* Dilute ’x’ μL of 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 -20°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
* Re-boil 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 appropriate amount of sample and 10µL of protein weight marker into gel
  + Place the lid on the tank and connect the leads to the power pack. 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)
* Add water, seal in plastic, and store in a refrigerator.