**Protein Extraction and Calibration Curve**

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

* With the larva constrained and folded in half, exposing the dorsum, make an incision through the cuticle at the proleg to extract hemolymph
* Pipet the hemolymph into 100μL of PBS
* Place the test tube into ice to slow oxidation and add inhibitor(s).
* **Store supernatant overnight at -80°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
  + Upto 7 treatments can fit on a plate
* Prepare three 100µl dilutions of each of your protein supernatant samples: 1X, 10X, 20X.
  + [diluting volume/#’x’ volume] = diluted volume
  + Ex: 100µl/1x = 30µl 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
* Mix each sample for 5 secs and Pipette 5μL of each standard or unknown sample into the appropriate microplate wells.
* Prepare 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
* Add 200μL of the Coomassie Reagent to each well.
* 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.
* 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:
    - µg/µl = 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
* 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.